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**INTERSPECIFIC HYBRIDISATION AND
MOLECULAR CHARACTERISATION OF HYBRIDS
IN THE GENUS *ZANTEDESCHIA***

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the requirements for the degree of
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Abstract

The genus *Zantedeschia* consists of two sections: a section containing *Z. aethiopica*, a white-flowered, evergreen species and a second section containing five winter-dormant species. A new species, *Z. odorata*, was recently described which does not fit into either of the two sections. Chromosome karyotypes of five species and two hybrid cultivars were prepared. Karyotypes are distinct between the two sections but not distinct within the second section. The karyotype of *Z. odorata* falls between the two sections although it is more closely related to *Z. aethiopica*.

Colchicine treatment of multiplying shoots *in vitro* produced tetraploid plants from eight cultivars and two species of the second section of the genus. Most of these plants were pure tetraploids. *Z. aethiopica* did not multiply *in vitro*. Colchicine treatment of *Z. aethiopica* 'Childsiana' germinating seed produced a few tetraploid plants and many diploid/tetraploid chimeric plants. A screening procedure for tetraploids using stomatal measurements, with confirmation by chromosome counting, was demonstrated to be an efficient and accurate way to identify tetraploids. Triploid plants were produced from two diploid/tetraploid crosses with the aid of *in vitro* embryo culture.

Crosses were made between the two sections using a number of species and genotypes at the diploid and tetraploid levels. In these crosses, endosperms were watery and transparent and embryos were small (in most cases less than 0.3 mm). Embryos embedded within endosperms were cultured because the embryos were too small to be cultured separately from the endosperms. From these cultures, over one hundred hybrid embryos were rescued. These hybrids were all albino. In an electron microscopy study, it was found that the plastids of these albino hybrids had no prolamellar body in the dark nor grana in the light.

Z. odorata was tested as a bridge for gene transfer between the two sections because it falls in between them. Hybridisation between *Z. odorata* and *Z. aethiopica* produced a number of virescent, albino and chimeric (green/albino) hybrids following embryo culture and seed germination. Hybrid production was much easier, however, when *Z. aethiopica* was used as the maternal parent. All hybrids rescued from crosses between *Z. odorata* and the second section of the genus were albino. This study also demonstrated that *Z. odorata* stigmas can receive pollen from the same spadix. *Z.*

odorata embryos become dormant before the seeds matured. Plants of *Z. odorata* did not produce any flowers unless they were treated with gibberellic acid (GA). However, two or three flowers per plant were produced when 50 ppm GA₃ or GA₄ + 7 was applied to tubers as a pre-planting treatment.

A partial library was constructed with total leaf DNA of *Z. aethiopica* 'Childsiana'. A species-specific nuclear DNA clone, pZAC3, was isolated by differentially screening this library with radioactively-labelled total DNA of different species. This clone was characterised by restriction enzyme mapping and RFLP (restriction fragment length polymorphism) analysis. By RFLP analysis, an apple rDNA clone differentiated between the sections of the genus. The apple rDNA clone and pZAC3 were successfully used for hybrid identification. From this library, six plastid (pt) DNA clones were also isolated by hybridisation with kiwifruit ptDNA clones. Using these ptDNA clones, RFLP bands were identified to differentiate between species and, in one case, between genotypes within a species. Biparental ptDNA inheritance and a ptDNA deletion were detected in the albino hybrids between the two sections with these ptDNA clones. The data from RFLP analyses gives the first molecular data on the phylogeny of *Zantedeschia* and indicates that *Z. odorata* is distinct from the previously-described two sections and falls in between the two sections.

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CHAPTER 1. General introduction

The calla lily (*Zantedeschia* spp) has been an important export flower crop from New Zealand over the last few years. The export value of calla lily cut flowers increased from a quarter of a million dollars in 1986 to two million dollars in 1990. The export value in 1990 exceeded all other export flower crops except for orchids. The genus *Zantedeschia* possesses two sections: a white-flower, evergreen species *Z. aethiopica* in section I and five winter-dormant species with coloured flowers in section II (Letty 1973). A newly-described species *Z. odorata* (Perry 1989) is not assigned to either of the two sections. Section II species, which provide most commercial cultivars, are very sensitive to *Erwinia* soft rot. *Z. aethiopica* shows some resistance to *Erwinia* and some other complementary characters to the coloured *Zantedeschia* species and cultivars such as a different flowering time and long flowering period. New cultivars with fine flower-colour, greater *Erwinia* resistance and/or an increased flowering period may be bred by combining the genetic characters of the two sections. Chi in 1990, however, showed that sexual hybridisation between the two sections was prevented by postfertilisation incompatibility.

The objectives of the present study were:

- (1) to test the effects of different species and genotypes on the crossability between the two sections of the genus,
- (2) to test the effects of ploidy level on the crossability between the two sections,
- (3) to test *Z. odorata* as a bridge line for gene transfer between the two sections,
- (4) to develop methods for hybrid identification and hybrid-plastome characterisation.

Overall, embryo culture was applied to rescue hybrids.

Literature on the relatively few studies in the genus *Zantedeschia* is reviewed in Chapter 2. Literature related to interspecific hybridisation in higher plants, including the types of incompatibility, approaches for overcoming the incompatibility and identifying hybrids, and plastid (pt) DNA inheritance, is reviewed in Chapter 3.

Descriptions of experimentation begin with chromosome karyotype analyses at Chapter 4 and tetraploid production at Chapter 5. Tetraploids produced were used for

interspecific hybridisation. Hybrid embryos were rescued from crosses between the two sections by embryo culture techniques (Chapter 6). Unfortunately, all hybrids rescued were albino. *Z. odorata* (Perry 1989) was tested as a bridge for gene flow between the two sections (Chapter 7). A number of nuclear and ptDNA clones were selected (Chapter 8) and successfully used for identification of hybrids and determination of ptDNA inheritance and ptDNA deletion (Chapter 9). Finally, a general discussion is presented (Chapter 10).

CHAPTER 2. The genus *Zantedeschia*

1. Classification of *Zantedeschia*

The genus *Zantedeschia* belongs to the Arum Family (*Araceae*) and was named by Sprengel in 1826 in honour of Professor Zantedeschi a botanist of Breschia. All species in this genus are native to central and southern Africa. The classification of *Zantedeschia* has been revised by Traub (1949) and Letty (1973).

The species of *Zantedeschia* have a number of common names (Traub 1949, Letty 1973). *Z. aethiopica* is commonly known as the arum lily. *Z. albomaculata* with its two subspecies *macrocarpa* and *valida* is commonly known as black-eyed arums. *Z. rehmannii* is called pink arum lily. *Z. pentlandii*, *Z. jucunda* and *Z. elliottiana* are all yellow arums. The name calla lily is now more commonly used in the literature for the pink and yellow types (Amos 1983, Tjia 1987, Corr & Widmer 1987, 1988).

The key to the species as described by Letty (1973) is listed in Appendix 1. Letty (1973) placed these species in two distinct but unnamed sections. In this thesis these two sections will be referred as to section I and section II. Section I contains only one species, *Z. aethiopica*, in which the plants are evergreen and the female flowers in the lower part of the spadix are interspersed with staminodes. The fruit turn orange in colour, become soft and later mucilaginous on maturity. Section II contains the remaining five species in which the plants are deciduous and the fruit remain firm and green until they finally wither and rupture or decompose on the ground. Letty (1973) stated that "there are no staminodes among the female flowers" in section II. Perry (1989) and Traub (1949), however, described that a few staminodes were interspersed with the female flowers in the upper part of the spadix in *Z. albomaculata*. The two sections also differ in spathe (flower) colour and flowering time (Table 1). *Z. aethiopica* generally has a white spathe, but green and pale pink forms are known in cultivation, and flowers from late winter to early summer. Section II includes a range of spathe colours and flowers from early to mid summer.

A new species, *Z. odorata*, was described in 1989 by Perry. According to Perry, *Z. odorata* falls in between the two sections. This species possesses some characters from each section (Table 1). It corresponds to *Z. aethiopica* (section I) in that the female flowers in the lower part of the spadix are interspersed with staminodes but is

similar to summer-flowering species (section II) in that the fruit remain firm and green until they finally wither on the ground. On the other hand, *Z. odorata* flowers in winter (late July and August in South Africa) and is dormant during summer. Furthermore, the flowering period, like the summer flowering species, is short in *Z. odorata*: approximately one month. *Z. aethiopica*, however, flowers for a much longer period: approximately six months, although the winter flowering *Z. odorata* overlaps with early flowering time of *Z. aethiopica*. A particular feature of this new species is that it invariably produces a delicate freesia-like scent (Table 1).

Table 1. Distinguishing characters in section I, *Z. odorata*, and section II (Based on Perry 1989 and Letty 1973)

	Section I	<i>Z. odorata</i>	Section II
Habit	evergreen	summer dormant	winter dormant
Flowering	Aug. - Jan.	Jul. - Aug.	Nov. - Jan.
Staminodes	numerous	many	few or none
Perfume	not scented	scented	not scented
Fruit	orange, soft	green, firm	green, firm
Spathe colour	white	white	coloured

2. Plant growth

In general, *Zantedeschia* is a perennial plant with a rhizome or tuber under ground. The plants of *Zantedeschia* will initiate flowers within three years from the time of planting when grown from seed, two years when grown from tissue cultured material, and 45 to 70 days when grown from two-year-old tubers (Tjia 1989). Plant growth is influenced by photoperiod. Plants grown under short photoperiod are shorter than plants grown under normal day length during the late spring and summer months. When the plants grown in late spring and summer are given supplemental lighting in the middle of the dark period, they will grow taller than plants grown in the same season but without a night break. Photoperiod, however, does not influence flowering in *Zantedeschia* (Post 1959, Tjia 1989).

After pollination and seed set, spathes progressively acquire a green pigmentation. They become leaf-like, both in colour and photosynthetic rates (Pais *et al.* 1979). The change in colour is believed to be induced by the action of endogenous hormones produced in the developing fruit and translocated to the spathes. The most important of these hormones has been identified as 6-(O-hydroxybenzylamino)9- β -D-ribofuranosylpurine, a naturally occurring cytokinin (Chaves Neves & Pais 1980a, b; Pais & Chaves Neves 1983).

There are several ways to propagate *Zantedeschia*. Some strains of *Zantedeschia* can be propagated uniformly from seed. For example, different colour selections of *Z. rehmannii* reproduce fairly true from seed (Harrison 1972). Moistened seed germinates in three to four weeks at 20 to 25 °C. Vegetative propagation can be carried out by division of the rhizome in *Z. aethiopica* or by cutting tubers in section II. Micropropagation by multiplying shoots *in vitro* (Cohen 1981) is now extensively used in New Zealand and overseas for rapid increase of new selections from section II.

***Z. aethiopica* (section I)**

The vegetative growth of *Z. aethiopica* is generally vigorous. Plants grow up to 120cm (Tjia 1989). *Z. aethiopica* 'Childsiana' is a dwarf cultivar with potential for pot culture (Welsh *et al.* 1988). Roots develop from almost all part of the rhizome and lateral buds mainly develop from the upper parts of the rhizome. Because *Z. aethiopica* is evergreen, harvested rhizomes can be replanted immediately, without dormancy.

Z. aethiopica flowers in winter and early spring and in some cases into early summer (Tjia 1989). The flowering period is from August to January in New Zealand. The flower is large. Flowering of *Z. aethiopica* depends on temperature, especially during the early summer months. Flower production drops when night temperatures do not fall below 15.5 °C. In Florida, where night temperatures seldom fall below 21 °C in the early summer months, *Z. aethiopica* ceases to flower altogether (Tjia 1989).

Meiosis of pollen mother cells occurs before the flower emerges (Earl 1957). No seed set when pollination in *Z. aethiopica* occurs less than two days before pollen dehiscence of male flowers in the same inflorescence whereas pollination between two days and 11 days before pollen dehiscence results in good seed set (New 1964). This indicates that the stigma loses pollen receptivity before pollen is released from male

flowers in the same inflorescence. Therefore emasculation is not necessary to prevent self-pollination.

Species in section II

Plants die down in late autumn and winter after the fruits mature (Letty 1973). In a growing season, tubers can develop several growing points. Consequently, growth during the next year will result in multiple-stemmed plants. If allowed to remain in the ground, a clump of plants will develop. Roots and contractile roots develop only from the upper part of the tuber. None are formed on the side and bottom of the tuber. If tubers are harvested at the end of the growing season and replanted immediately they are slow to resume growth (Tjia 1989). Storage is needed to break the dormancy. Good storage conditions can increase plant height and number of leaves and shoots per tuber in *Z. rehmannii* and *Z. elliottiana* (Corr & Widmer 1988).

Plants of section II flower in the summer. High night temperatures do not affect inflorescence development but do affect colour development in the spathe. Red and pink coloured spathes have a bleached appearance when grown at high night temperatures (Tjia 1989). Self-fertilisation by pollen from the same inflorescence occurs readily in plants of summer-flowering species. Emasculation must be done in advance of pollen dehiscence to prevent self-pollination. Stigmas may be pollinated as soon as the inflorescences are emasculated (New 1964).

Growth regulators have been applied for controlling the growth and flowering of *Zantedeschia*. Flowers were induced from tubers, 2 cm diameter, when they were dipped into a solution of 40 ppm GA₃, drained and planted (Cohen 1981). Soaking the tubers of *Z. elliottiana* and *Z. rehmannii* in 500ppm GA₃ prior to planting increased number of flowering shoots and flowers per shoot. Leaves on the treated plants were more narrow, but length was unaffected (Corr^{and Widmer} 1987). Paclobutrazol at 4 mg/pot was used effectively on *Zantedeschia* to control plant height (Tjia 1987)

Z. odorata

It was noted that plants of *Z. odorata* rarely showed flowers. From more than 100 plants in cultivation for five years, only one inflorescence had been produced (Perry 1989).

3. Pests and diseases

Zantedeschia are remarkably tolerant and relatively free from most pests and diseases. However, plants of section II suffer from a tuber soft rot disease caused by the bacterium *Erwinia*. This disease spreads through the plants especially in wet or heavy soil (Action Flower Export Co. Ltd. Feb. 1987). *E. carotovora* is thought to be the predominant pathogen and infects through wounded tissue. No satisfactory method has been developed for control this disease. *Z. aethiopica* is relatively tolerant to *Erwinia*. Apart from tuber soft rots, disease outbreaks on flowers and foliage are rare. Various diseases, such as, crown rot, leaf spots, root rot, storage rot, and tomato spotted wilt virus (Pirone 1970) have been recorded on *Zantedeschia*, but they are not severe problems.

4. Breeding

A number of crosses were made using *Z. rehmannii* Engl, *Z. albomaculata* Baill, and *Z. elliottiana* Engl. by Shibuya (1956). Observations were made on F1, F2, F3 plants and progenies of backcrosses. A large amount of data were presented on the segregation of a number of characters in the hybrids. From these data, two major conclusions were drawn. First of all, the occurrence and expression of anthocyanin (pink) colour in *Zantedeschia* depends on the complementary effect of four pairs of dominant genes which were named as A, B, C, D. Secondly, the yellow or golden colour expression is controlled by two duplicate genes (Y1, Y2) and two complementary genes (Ia, Ib) which suppress the expression of the yellow colour caused by Y1 and Y2. In addition, the seeds of crosses involving *Z. elliottiana* germinated poorly and generated a considerable number of weak plants and albinos.

Crosses within section II of *Zantedeschia* produced virescent hybrids (New & Paris 1967). Virescence is a condition in which the first leaves of affected plants are markedly chlorophyll deficient and can be mistaken for albino, but under favourable conditions, gain chlorophyll as they age. Interspecific-reciprocal crosses and self-pollinations were made using *Z. elliottiana*, *Z. rehmannii*, *Z. albomaculata*, and a *Zantedeschia* hybrid 'Apricot Sunrise'. Virescence was expressed in the cross *Z. elliottiana* X *Z. rehmannii*, but not in the reciprocal crosses. There was no virescence in reciprocal crosses between *Z. elliottiana* and *Z. albomaculata* but there was

virescence in reciprocal crosses between *Z. rehmannii* and *Z. albomaculata*. Albino hybrids were also found in some crosses. The segregation of leaf colour (green, virescence and albino) was complicated in backcrosses and F2 progenies. This segregation was unlikely to be controlled by a single recessive gene or by a simple cytoplasmic-nuclear gene relationships. Temperature and light modified the expression of virescence.

New Zealand has had a number of amateur *Zantedeschia* breeders. They have bred coloured *Zantedeschia* hybrids which are attracting considerable interest overseas (Amos 1983). Whiteley (in Auckland) was one of the earlier hybridisers of *Z. elliotiana* and *Z. rehmannii*. Harrison (in Palmerston North) was one of the most successful calla lily breeders. He has bred some outstanding calla lilies in a range of sizes and colours. In 1983, many of his selections were named and released. Other calla lily breeders were T Brljević (in Auckland), T Brljević (in Maungaturoto), T Hatch (in Pukekohe) and J Matthews (in Waitara) (Amos 1983).

Hybridisation between the two sections has never led to viable progeny. Early studies showed no seed formation after cross-pollination (Traub 1949). A recent study (Chi 1990) demonstrated that incompatibility between the two sections occurs after fertilisation. Most embryos from intersectional crosses only developed to a stage comparable with the globular or pre-torpedo stage of selfed embryos. The arrest of embryo growth in incompatible crosses was accompanied by degeneration of the endosperm. Most embryos (dissected six to ten weeks after pollination) were not elongated (0.1 - 0.2 mm). These embryos remained globular on culture medium. Three embryos which were elongated (0.7mm) when dissected, eventually produced albino shoots through embryo culture.

CHAPTER 3. A literature review of interspecific hybridisation in plants

Interspecific hybridisation has contributed to the improvement of some crop cultivars and has potential to improve a number of other crops. Hybridisations between species within a genus may be compatible or incompatible. Incompatibility may occur before fertilisation and/or after fertilisation. Some interspecific hybrids reveal an abnormality in their vegetative development or their reproductive development, such as chlorophyll deficiency of leaves and sterile pollen. This abnormality is also considered to be a type of incompatibility. A number of methods have been used to overcome incompatibility. Only a few putative hybrids are normally rescued from an incompatible cross even though a large number of pollinations are made. It is always necessary to test whether these potentially valuable plants are truly hybrids before they are used further. This chapter reviews literature which is related to interspecific hybridisation in higher plants, including the types of incompatibility, methods for overcoming incompatibility and identifying hybrids, and patterns of plastid DNA inheritance.

1. Incompatibility in plant interspecific hybridisation

1.1. Prefertilisation incompatibility

Prefertilisation incompatibility is a pollen-pistil rejection response involving reduced pollen germination, slow pollen tube growth or distortion or bursting of pollen tubes (Evans 1962b, Hovin 1962b, Chen & Gibson 1972, Perez & Moore 1985). This incompatibility varies according to the genotypes of the individual plants that are crossed (Evans 1962b). Prefertilisation interspecific incompatibility may also vary according to the direction of the cross (Hovin 1962b, Smartt 1980). This is known as unilateral incompatibility. Studies with *Prunus* species have shown correlations between pollen tube growth rate, pollen size and pistil length such that pollen tubes from one species with short style and small pollen will be unlikely to achieve fertilisation of a long-styled species (Perez & Moore 1985). This provides a basis for unilateral incompatibility in addition to genetic control factors.

1.2. Postfertilisation incompatibility

Postfertilisation incompatibility includes zygotic or early embryonic inviability and disturbance of the embryo-endosperm-maternal tissue nutritional balance within the ovule, leading to endosperm failure or hypertrophy of maternal tissue at the expense of the embryo. The intensity of postfertilisation incompatibility frequently varies according to the direction of the cross (Price & Stutz 1974, Mok *et al.* 1978, Rabakoarihanta *et al.* 1979, Shii *et al.* 1982, Pandey *et al.* 1987) and the genotype of the parents (Evans 1962a, Hovin 1962a, Mok *et al.* 1978, Chen *et al.* 1983).

The failure of endosperm leading to death of a potentially viable hybrid embryo is a common result of crosses between closely related species in many genera studied (Williams 1987, Williams *et al.* 1987a), as well as in *Zantedeschia* (Chi 1990). The endosperm is important to the development of embryos because not only does it supply nutrients to the embryo but it also regulates the water potential environment of the embryo. Endosperm generally has a lower water potential at early embryo development stages than at later embryo development stages (Smith 1973). During early embryogeny in *Phaseolus vulgaris*, the liquid endosperm had the lowest osmotic value (most negative), followed by the embryo, the seed coat and the pod tissues (Yeung & Brown 1982).

The reason for endosperm abortion in incompatible crosses is not clear. However, a number of hypotheses have been made including restricted nutrient flow from maternal tissues, genetic imbalance within the endosperm or a direct effect of the hybrid embryo on endosperm development.

Cooper and Brink (1942) attributed endosperm abortion in *Nicotiana rustica* X *N. glutinosa* to unsuccessful competition with the maternal tissue for nutrients. For crosses of *Hordeum jubatum* X *Secale cereale* (Brink & Cooper 1944, Cooper & Brink 1944) and *Elymus virginicus* X *Agropyron repens* (Beaudry 1951), it was suggested that endosperm growth may be limited by a restricted nutrient flow through abnormal antipodal cells. In the incompatible crosses of *Zantedeschia* species, early necrosis at the chalazal end of the embryo sac may be responsible for the degeneration of the endosperm tissue because the chalaza appears to play an important role in transporting sucrose to the endosperm (Chi 1990).

Thompson and Johnston (1945) suggested that endosperm abortion was caused either by a genetic imbalance in the genome of the endosperm itself or by an unfavourable reaction between it and the surrounding maternal tissues. These views were supported by Chen and Gibson (1971). The genetic imbalance hypothesis was supported by many authors and developed into an endosperm balance number (EBN) hypothesis (Johnston *et al.* 1980, Johnston and Hanneman 1980), as described later in Section 2.3.

A developing embryo may contribute to endosperm failure. Embryoless seeds have normal endosperm, while in those containing an embryo, the endosperm invariably degenerates in crosses of *Gossypium hirsutum* X *G. arboreum* (Weaver 1957) and *Trifolium nigrescens* X *T. occidentale* (Chen & Gibson 1974). It is not clear, however, how the developing embryo inhibits the growth of the endosperm. Incompatible interaction of maternal and hybrid tissues may cause disturbances in early seed nutrient and water transfer patterns (Williams & White 1976). The endosperm may, in fact, fail to compete with the embryo for nutrients. A reciprocal type of nutrient transfer disturbance is suggested by observation of an abnormally thick layer of endosperm surrounding underdeveloped hybrid embryos in the cross *Phaseolus coccineus* X *P. vulgaris* (Shil *et al.* 1982).

1.3. Plastome/genome incompatibility

Albino and chlorophyll-deficient hybrids have been produced from interspecific crosses in many genera, as *Trifolium* (Przywara *et al.* 1989), *Impatiens* (Arisumi 1985) and *Zantedeschia* (New & Paris 1967, Chi 1990). In some crosses albino progeny are present in one crossing direction but not in the reciprocal cross. The hybrids between *Trifolium repens* and *T. nigrescens* or *T. uniflorum* are good examples of such unilateral abnormality (Hovin 1962a, Pandey *et al.* 1987). In other crosses, albino progeny are produced in both crossing directions, for example, in crosses between *Trifolium repens* and *T. hybridum* (Przywara *et al.* 1989).

The level of chlorophyll deficiencies in interspecific hybrids is variable, depending on the plant species used in a cross. In some cases only total albinos and albinos with green sectors hybrids are produced (Przywara *et al.* 1989). In other crosses both green and albino hybrids are produced (Arisumi 1985). Much work has been done to achieve green hybrids when albinism is a feature in the crosses, but few reports have

studied the mechanism of the formation of albino hybrids. In general, most researchers believe that the incompatibility between the plastome (plastid genome) and the hybrid genome causes the failure of chloroplast development in the hybrids. This has been demonstrated in the genus *Oenothera* (Stubbe 1964). Five types of plastome and six types of genome have been classified. Each type of plastome matches with certain genome(s). If the plastome does not match the hybrid genome, albino and/or variegated plants will be produced. Mechanism(s) for plastome and genome incompatibility is unknown.

In the genus *Zantedeschia*, many green hybrids have been produced from interspecific hybridisations within section II. However, with some crosses many albinos develop (New & Paris 1967). Only albino hybrids have been produced from interspecific hybridization between the two sections of *Zantedeschia* (Chi 1990).

1.4. Hybrid sterility

Hybrid sterility is a common problem in interspecific hybridisation. Sterility varies from almost fully sterile to almost fully fertile. Wide variation is observed not only in different interspecific combinations but also in different F1 derivatives of the same combination (Williams & Verry 1981, Chen *et al.* 1983, Pandey *et al.* 1987) and in different individual descendents of the same F1 isolate (Smartt & Haq 1972, Williams & De Lautour 1981, Williams *et al.* 1987b). Parental genotypes may have a marked effect on hybrid fertility (Chen *et al.* 1983). The degree of sterility is not necessarily related to the ease of producing the hybrids. The cross *Phaseolus coccineus* X *P. vulgaris* requires embryo culture but produces hybrids with 81% fertility. In contrast, the reciprocal cross, although giving normal seed development, produces hybrids with only 27% fertility (Shii *et al.* 1982).

Sterility in interspecific hybrids may be chromosomal and/or genetic. Chromosomal sterility may be associated with gross meiotic abnormalities resulting from failure of distantly related genomes to pair, from the presence of uneven numbers of genomes in interploid crosses, or from the presence of a different basic number. Genetic sterility may occur, even in the presence of effective chromosome pairing, as a result of physiological disturbance of reproductive development, or of cryptic genetic and structural differences between homoeologous chromosomes (Stebbins 1971, Williams *et al.* 1982).

Certain hybrid populations may show substantial increases in pollen fertility in successive generations (Grant 1966a, b). The increase in fertility may be accompanied by decreasing interfertility with parent species, as in *Phaseolus vulgaris* X *P. coccineus* (Smartt & Haq 1972) or may not be accompanied by decreasing interfertility, as in *Ornithopus sativus* X *O. compressus* (Williams *et al.* 1987b). Functional fertility of F1 hybrids in backcrosses may differ according to which of the parent species is involved. A higher frequency of embryos was recovered in backcrosses if the original male parent of the hybrid was used again as the male parent in lilies (Asano 1980) and in *Phaseolus* (Rabakoarihanta *et al.* 1980). These suggest that the relative dosages of genetic material from the two species may be important in the regulation of normal embryo development. For some hybrids, such as hybrids of *Trifolium* (Williams *et al.* 1982, Pandey *et al.* 1987) and in lilies (Asano 1978), fertility may decrease in backcross progenies.

2. Approaches for overcoming interspecific incompatibility

Different approaches have been used to overcome interspecific incompatibility (as reviewed from Section 2.1 to Section 2.5). Certain approaches may be suitable only in certain crosses. In some crosses, two or more approaches need to be combined to achieve interspecific hybrids. This was found in attempts to hybridise *Nicotiana repanda* with *N. tabacum* (Shintaku *et al.* 1988).

2.1. Selection of potential parent plants

Interspecific incompatibility varies in intensity depending on the genotypes of individual plants and the direction of the crosses. Selecting suitable genotypes and an appropriate cross direction with better compatibility saves time and increases the chances of achieving hybrids.

To examine prefertilisation compatibility, an effective method is to monitor the extension of pollen tube growth in reciprocally cross-pollinated pistils of a few individuals of the two parent species. Crosses between a self-compatible and a self-incompatible species are more likely to be successful if the self-compatible species is used as the female parent (Matzk 1980), although exceptions do occur. A correlation between graft compatibility and pollen tube growth in interspecific crosses was found

in *Trifolium* (Evans 1960). To examine postfertilisation compatibility, one method is to make a series of crosses using a number of genotypes of two parents. At a suitable interval after pollination, ovules are then dissected and scored for extent of embryo and endosperm development. By this method, Taylor *et al.* (1983) found that the genotype had a significant effect in the development of hybrid embryos between *Trifolium sarosiense* and *T. pratense*.

It is sometimes possible to choose potential parents based on the genetic relationships among species. More closely related *Trifolium* species can be hybridised more readily than less closely related species (Taylor *et al.* 1963). Chromosome karyotype analysis has been used to study the genetic relationships among plant species and may be useful in the selection of a potential parent. It is also possible that parents with improved compatibility may be identified using the newer DNA-analysis techniques such as DNA finger printing (Ryskov *et al.* 1988; Rogstad *et al.* 1988), restriction fragment length polymorphism (RFLP) (Tanksley *et al.* 1989), and random amplified polymorphism DNA (RAPD) markers (Williams *et al.* 1990).

2.2. Embryo culture

The embryo was the first plant organ to be successfully cultured *in vitro* in 1904. After about 20 years of research, two important practical applications of embryo culture were made: the rescue of interspecific hybrid embryos and the propagation of orchids by *in vitro* germination of their small and physiologically immature seeds. Now, embryo culture techniques are widely used in crop improvement with emphasis on rescuing interspecific hybrid embryos (Williams *et al.* 1987a). Interspecific hybrids that would otherwise be lost in aborting seeds have been rescued by embryo culture technique (for reviews see, Rangan 1982, 1984; Collins & Grosser 1984; Williams 1987, Williams *et al.* 1987a). In some crosses, where embryo culture has not been successful, ovule culture has been used.

Media and culture conditions

Medium requirements for embryos depends on the degree of maturity at excision since embryo development within the seed involves a progressive change from total dependence on surrounding tissues to complete independence at germination. Nutrient and osmotic value are the two most important factors in a medium.

Mature or nearly mature embryos require only basic mineral salts and an energy source such as 2% or 3% sucrose. Immature embryos, however, require a more complex array of organic additives. These requirements are still poorly defined with respect to precise metabolic function and the time span for which they are needed. Such additives have included B vitamins, hormones, amino acids, amino nitrogen, purines, or undefined sources of some of these components (e.g. casein hydrolysate, coconut water, yeast extract, or various plant extracts). Use of these additives are generally based on trial and error rather than definitive studies. Detailed studies of the physiology and *in vitro* requirements of normal immature embryos may improve rates of recovery of interspecific hybrid embryos. They would also help to extend the range of developmental stages that can be rescued down to immature, globular pro-embryos, and perhaps eventually to the zygote itself.

The optimal osmotic value of the medium depends on the age of the embryo at excision, and one value will not necessarily give satisfactory growth from the very immature stage to germination. The liquid endosperm in which young embryos are constantly bathed has a low (more negative) osmotic value and work on *in vitro* embryo culture indicates that media with low osmotic values are beneficial to the growth of young embryos (Yeung & Brown 1982). In one study (Niederwieser 1990), it was found that pro-embryos of *Ornithogalum* required a medium with relatively high sucrose concentration (70 g/l) (low osmotic value) for embryogenesis but after 14 days they had to be transferred to medium with low sucrose concentration (10 g/l) for germination and seedling growth.

Charcoal is occasionally added in embryo-culture medium to absorb toxic metabolites (Fridborg *et al.* 1978, Cade *et al.* 1990).

Relatively little systematic experimental work has been done to investigate culture conditions such as temperature, light, or the gaseous phase within the culture vessels. Embryos have most commonly been cultured at temperatures from 20-30 °C in light, although some workers have routinely incubated cultures in the dark and then transferred them to light for germination.

Modifications of embryo culture

To approximate the change of osmotic potential within the embryo sac during development, a serial transfer of embryos from complex media with high osmotic potential to progressively simpler media with lower osmotic potential has sometimes been used (Mauney 1961; Phillips *et al.* 1982). An alternative method is to culture embryo on a small area of medium in which the concentration of components is changed by diffusion into the surrounding medium of lower concentration (Monnier 1978).

The transplanted nurse endosperm technique was successfully used to rescue hybrid embryo in pasture legume genera (Williams & De Lautour 1980). At a suitable stage, the hybrid embryo was inserted into a normal developing nurse endosperm in which the normal embryo was discarded. The role of the transplanted endosperm has not been experimentally defined, but it is possible that the transplanted normal endosperm supplies a nutritional or hormonal requirement to hybrid embryos between the late globular to early heart stage.

Ovule culture is the preferred method when embryos are unable to develop beyond the globular stage (Lagriffol & Monnier 1985, Williams 1987). Transition from the globular to heart stage of embryo development marks the initiation of cotyledons and bipolar growth. This transition appears to be a natural breakpoint for the culture of excised embryos (Tilton & Russell 1984), as there have been few reports of successful culture from the globular stage (Neal & Topoleski 1983, 1985). Presumably, the induction of bipolarity is normally dependent on asymmetry of the embryo's attachment within the ovule, and on a critical gradient of growth regulators. Interspecific hybrids have been rescued by culture of young embryos in partial ovules packed with transplanted nurse endosperm in *Ornithopus* (Williams & De Lautour 1981), in half ovules in liquid medium *Brassica* (Harberd 1969), and in whole ovules for a short period followed by culture of the separated, partial mature embryos (ovule-embryo culture) in legume genera (Cohen *et al.* 1984, McCoy 1985, Williams *et al.* 1987b; Przywara *et al.* 1989).

Regenerating multiple plants from a hybrid embryo has been used to obtain viable hybrid plants or to increase the efficiency of hybrid recovery. Young hybrid embryos of *Trifolium* produced callus which subsequently regenerated multiple plantlets by organogenesis or somatic embryogenesis (Rupert *et al.* 1979). Through a callus phase, the efficiency of hybrid recovery was increased in crosses between *Lycopersicon*

esculentum and *L. peruvianum* (Thomas and Pratt 1981). In *Zantedeschia*, multiple shoots were regenerated from haustorial callus of hybrids between the two sections (Chi 1990). In interspecific crosses of *Rubus* (Fiola & Swartz 1985), a clone of plantlets from each embryo was obtained after the embryonic axes were separated from their cotyledons and cultured on shoot induction and proliferation media. One of many plants regenerated from hybrid leaves survived and flowered (Iwai *et al.* 1985) although hybrid seedlings which were rescued through ovule culture died before 3-4 leaf stage, in cross *Nicotiana repanda* X *N. tabacum*.

2.3. Manipulation of endosperm balance number (EBN)

Double fertilisation is a feature common to all angiosperms. Endosperm is formed by fertilisation of the two polar nuclei of the embryo sac by a sperm nucleus. The endosperm is triploid (3x) in crosses between diploids since it receives two sets of maternal chromosomes and one set of paternal chromosomes. After fertilisation, the endosperm develops rapidly, becoming the nutritional tissue for the embryo. The survival of the embryo depends on normal development of the endosperm in almost all angiosperm species.

Endosperm balance number (EBN) is an effective ploidy level that determines the crossing behaviour of a species. For an intra- or interspecific cross to be successful, the endosperm must have a 2 maternal EBN : 1 paternal EBN ratio. If the ratio departs from this figure, the endosperm breaks down during development. The EBN of a species may not be a direct reflection of its ploidy. For example, ^{for} different species of *Solanum*, the EBN has been determined as follows: 4x(2EBN or 4EBN), 3x(3EBN or 2EBN) and 6x(4EBN) (Johnston *et al.* 1980). Doubling of chromosome number also doubles the EBN (Bamberg & Hanneman 1990). Matching EBN is a necessary but not a sufficient condition for a successful cross. Successful crosses in species with the same EBN may be prevented by prefertilisation barriers unrelated to EBN. In addition, crosses between species differing in EBN can be successful, albeit at a very low rate (Parrott & Smith 1986).

Ehlenfeldt and Hanneman (1988) investigated the genetic control of EBN in *Solanum commersonii* (2x, 1EBN) and *S. chacoense* (2x, 2EBN). They concluded that: (1) three un-linked loci control the system; (2) the loci are homozygous within a species; (3) the genes have additive effects and are of equal strength within a species; (4) the genes

within *S. chacoense* have twice the effect with respect to endosperm regulation as those within *S. commersonii*; (5) a slight excess maternal dosage will produce the qualitative effect of small but viable seed.

When two parents have unlike EBNs, the EBN ratio of hybrids can be manipulated to 2 maternal EBN : 1 paternal EBN by chromosome doubling (Bamberg & Hanneman 1990), using 2n gametes (Ehlenfeldt & Hanneman 1984, Brown & Adiwilaga 1990), or reducing the ploidy level of a polyploid parent (Yerk & Peloquin 1990). Interspecific hybrids have been obtained by manipulation of EBN in *Solanum* species (Johnston *et al.* 1980, Johnston & Hanneman Jr 1982, Ehlenfeldt & Hanneman Jr 1984, Brow & Adiwilaga 1990, Bamberg & Hanneman 1990, Yerk & Peloquin 1990), *Trifolium* species (Parrott & Smith 1986), *Medicago* species (McCoy & Smith 1984), *Glycine max* (Zhang & Palmer 1990), maize (Lin 1984), and *Impatiens* species (Arisumi 1982). Some unsuccessful EBN manipulations have also been reported, such as in *Cyphomandra* (Pringle & Murray 1991).

2.4. Somatic hybridisation

Somatic hybridisation is a powerful way to overcome sexual incompatibility. It is especially useful for overcoming prefertilisation incompatibility because it does not involve pollination and fertilisation. Since the first success of protoplast fusion (Carlson *et al.* 1972), people have applied this technique for crop improvement. In early protoplast fusion studies, workers concentrated on performing very wide hybridisations. In these wide combinations, selective elimination of one or other parental chromosomes occurred and regeneration capability was either reduced or absent. Plants which were regenerated from those hybridisations were mostly infertile, thus precluding their integration into crop improvement programs (Bravo & Evans 1985).

Problems associated with somatic hybridisation subsequently led researchers to concentrate on producing hybrids between closely related species. Many successes have been achieved. For example, numerous citrus somatic hybrids have been reported from combinations between sexually compatible and sexually incompatible species (Grosser & Gmitter 1990, Grosser *et al.* 1992) and fertile somatic hybrids have been obtained between navel orange (*Citrus sinensis*) and Troyer citrange (*C. sinensis* X *Poncirus trifoliata*) (Ohgawara *et al.* 1991). Many studies have attempted to improve crops via protoplast fusion through production of cybrids (cytoplasmic hybrids) for

transfer of agriculturally useful traits that are cytoplasmically encoded (Pelletier *et al.* 1983, Jarl & Bornman 1988) or through production of irradiated asymmetric hybrids for transfer of a few nuclear genes (Gupta *et al.* 1984, Imamura *et al.* 1987, Gleba *et al.* 1988).

Procedures for plant somatic hybridisation include protoplast isolation, protoplast fusion, heterokaryon selection, hybrid plant regeneration and hybridity confirmation. Mechanical means produce protoplasts with poor viability in low yield. The enzymatic method which was first described by Cocking (1960) is the preferred method for rapid isolation of viable protoplasts in high yield (Evans & Bravo 1983). Polyethylene glycol (PEG) was first used to fuse protoplasts by Kao and Michayluk (1974) and is now widely used as a chemical fusogen. Electrofusion has several advantages over chemical fusion, such as rapidity, higher fusion frequency, and not using harmful chemicals for protoplast fusion of *Solanum spp* (Fish *et al.* 1988). Since the fusion is nonspecific, genetic complementation (Imamura *et al.* 1987, Gleba *et al.* 1988, Ratushnyak *et al.* 1991), hormone-free medium (Carlson *et al.* 1972) and microisolation (Kao 1977) are used to select the desired heterokaryons. Even though plants are generated from selected cells, it is still necessary to confirm the hybridity. Intermediate morphology, chromosome number, chromosome morphology, isozyme patterns, and DNA analyses are employed for this purpose.

One major disadvantage of somatic hybridisation is the difficulty in regenerating plants from protoplasts of most major crop species. The advantages are: easily overcoming prefertilisation incompatibility and making extensive recombinations among nuclear genome and cytoplasmic genome. Twelve types of recombinations among nuclei, mitochondria and chloroplast are summarised by Bravo and Evans (1985). An agronomically important example in *Brassica* has been incorporation into one plant of the chloroplast encoded triazine resistance from one parent with the mitochondria encoded male sterility from another parent (Barsby *et al.* 1987).

2.5. Other approaches

Irradiation has been used to overcome incompatibility in somatic hybrids between *Physalis minima* and *Datura innoxia* (Gupta *et al.* 1984), between carrot and tobacco (Dudits *et al.* 1987), and in sexual hybridisation between *Nicotiana repanda* and *N. tabacum* (Shintaku *et al.* 1988). It is considered that the chromosome and/or

chromosomal fragments bringing about hybrid inviability is eliminated from one parent genome after destruction of chromosomes with ionizing radiation. The frequency of seedling production in irradiated pollinations was significantly higher than from non-irradiated pollinations between *Trifolium repens* and *T. hybridum* although the seedlings died after transferring to soil (Przywara *et al.* 1989)

In vitro pollination and fertilisation has been used to overcome prefertilisation incompatibility (Collins *et al.* 1984; Deverna *et al.* 1987). Burk (1967) has transferred germplasm from a disease resistant wild species (*Nicotiana repanda*) through an intermediate bridge species (*N. sylvestris*) into a cultured species (*N. tabacum*). A bridge line has been used to assist the production of sexual hybrids between *Lycopersicon esculentum* and *Solanum rickii* (Deverna *et al.* 1990).

Hybrid fertility may be improved by doubling chromosome number of the hybrid (Apparao & Ramavarma 1974), or the parents (Taylor *et al.* 1963) if the parental species are different in chromosome morphology. If the parental species are different in ploidy level, the fertility of the hybrid may be improved by doubling chromosome number of one parent with lower ploidy level or the chromosome number of the hybrid. Chromosome doubling does not necessarily restore full fertility immediately if genetic sterility is also present, as seen in a hybrid of *Ornithopus pinnatus* X *O. sativus* (Williams & De Lautour 1981). The F₁ hybrids of *Phaseolus vulgaris* X *P. coccineus* showed a mean fertility of 21%; fertility rose to only 42% after chromosome doubling but reached 66% in the C₅ generation (Smartt & Haq 1972).

3. Hybrid identification

It is necessary to determine whether wide hybrids contain the genomes from both parents. One genome may have been eliminated (Bennett *et al.* 1976), the progeny may have arisen by parthenogenetic development (Heslop-Harrison 1972), or the eggs may have been fertilised by stray pollen. Confirming hybridity at an early stage is desirable.

Morphological and quantitative characters alone may be insufficient or inappropriate for confirming hybridity if the parent species are similar or if one parent species carries major epistatic factors (Williams 1987). Even though some morphological

characters are very useful for hybrid identification in mature plants, they may not be useful in young seedlings. In addition, the expression of morphological markers is unreliable because changes in morphology are easily influenced by tissue culture techniques involved in interspecific crosses (Weeden 1989). Reduction of hybrid fertility is sometimes an indicator for hybridity but it is also not reliable. Chromosome morphology may be used to identify hybrids for plant species with large chromosomes but it is rarely satisfactory for species with small chromosomes. The best confirmation of hybridity is provided by unequivocal transmission of distinctive qualitative characters, such as isozymes (Weeden 1989) and DNA markers, from the male parent.

3.1. Isozyme techniques

For nearly three decades, plant breeders have had the opportunity to incorporate isozyme techniques into their research. Isozyme techniques are widely used in plant breeding such as cultivar identification, somatic clone variation analysis, gene mapping, polyploid level analysis, as well as confirming hybridity (Weeden 1989).

In a broad sense, the term isozyme (also called isoenzyme) refers to any two distinguishable proteins that catalyse the same biochemical reaction (Weeden 1989). Although seven types of isozymes have been described (Markert and Whitt 1968), most of these classes are rarely encountered. The class of isozymes produced by allelic polymorphism at the structural gene is very common and has been given the separate designation "allozyme" (Prakash *et al.* 1969). The minimum number and the subcellular location of isozymes of each enzyme in higher plants is conserved (Gottlieb 1982).

The principle of isozyme analysis is to separate isozymes on the basis of charge or size through gel electrophoresis, and subsequently to visualise the enzymatic bands by adding a substrate which can generate colour through enzymatic reaction.

Starch gels are most commonly used in plant genetics and breeding research in preference to polyacrylamide gels (Cardy *et al.* 1980, Arus & Orton 1983, Shields *et al.* 1983, Soltis *et al.* 1983, Cardy & Beversdorf 1984, Weeden 1984, Kahler & Lay 1985). It has at least four advantages: simplicity of technique and apparatus, nontoxicity of material, low equipment cost, and the ability to slice the gel. Starch gel can be sliced

horizontally into a number of duplicate slabs. Each slab may then be assayed for different enzyme systems (Weeden 1989).

Polyacrylamide gel electrophoresis (PAGE) gives better resolution than starch gel electrophoresis, but it is more expensive and the chemicals are toxic. Isoelectric focusing gives good resolution but requires more expensive reagents and equipment.

To identify hybrids, a distinguishable isozyme pattern between two parents needs to be defined. A true hybrid should codominantly express the allozyme from the male parent. For example, in crosses between *Trifolium repens* and *T. hybridum*, the electrophoretic banding patterns of leaf isozymes of peroxidase in the F1 seedlings enabled the identification of parental bands, giving evidence of hybridity (Przywara *et al.* 1989). Isozyme phenotypes have been widely used for identifying hybrids from wide crosses and protoplast fusion. This application has recently been reviewed by Weeden (1989).

3.2. Molecular biology techniques

The recent development of DNA technology has been employed to confirm hybridity between distant crosses. Detection of species-specific or genomic-specific DNA and analysis of restriction fragment length polymorphism (RFLP) are two main approaches used for hybrid identification.

Detection of species-specific DNA

Species specific repetitive DNA was first used to identify interspecific somatic hybrids between *Hyoscyamus muticus* and *Nicotiana tabacum* (Saul & Potrykus 1984), and more recently between *Lycopersicon esculentum* and *Solanum acaule* (Schweizer *et al.* 1988), and between *Solanum brevidens* and *S. tuberosum* (Pehu *et al.* 1990). The hybridity is confirmed if species specific DNA sequences from both parents can be detected in the putative somatic hybrid. Although many methods can be used to isolate species specific DNA sequences, three methods are most common.

In the first method, genomic DNA from two species are digested by a restriction enzyme, and the restricted fragments are separated by gel electrophoresis. If a

prominent band is found in one species but not in another, DNA in this band will be cloned. Clones containing species specific DNA sequences will be identified by Southern hybridisation. This method was used to isolate *Lycopersicon esculentum* specific repetitive DNA sequence (Schweizer *et al.* 1988) and genome-specific repetitive sequences in the genus *Oryza* (Zhao *et al.* 1989).

In the second method, a partial genomic library is screened by radioactively labelled nuclear DNA from a different species. If any colony shows signal for one species probe, but not for the another, it will be isolated as a putative species specific clone. The specificity is confirmed by Southern hybridisation. This method was successfully used to isolate species specific repetitive DNA sequences in *Hyoscyamus muticus* and *Nicotiana tabacum* (Saul & Potrykus 1984), in *Solanum acaule* (Schweizer *et al.* 1988), in *Beta procumbens* (Schmidt *et al.* 1990), and in *Solanum brevidens* and *S. tuberosum* (Pehu *et al.* 1990)

The third method was used to clone male-specific DNA sequences of mouse (Lamar and Palmer 1984). DNA from female mice was sheared by sonication and DNA from male mice was completely digested with a restriction enzyme. A 100-fold excess of sheared female DNA was mixed with enzyme-digested male DNA and the mixture was denatured and annealed. Double-stranded DNA was isolated by hydroxylapatite chromatography. Three types of double-stranded DNA was formed. In type I both strands were derived from sheared female DNA. In type II one strand was derived from sheared female DNA and the other strand was derived from enzyme-digested male DNA that was homologous with female DNA. In type III both strands were derived from enzyme-digested male DNA that shared no homology with female DNA. A small proportion of male DNA that was homologous with female DNA may form type III double-stranded DNA. Only type III double-stranded DNA can be easily cloned because only these DNAs contained two enzyme-digested ends which can be ligated into a vector. A library constructed from these procedures would be enriched for male-specific clones.

The yield of clones from Lamar and Palmer's procedure was low, since it depended on the slow annealing of diluted male ("tester") DNA. To improve the yield of clones, the annealing of the tester DNA was speeded up by using a formamide-phenol emulsion reassociation technique (F-PERT) (Kohne *et al.* 1977) in modified procedures of Kunkel *et al.* (1985) and Casna *et al.* (1986). Using the modified method, a genomic-

specific DNA clone was isolated in *Actinidia deliciosa* var. *deliciosa* (Crowhurst & Gardner 1991).

These three methods have their own advantages and disadvantages. The first method is simple. But the usefulness is restricted if no prominent band can be detected. The second method involves screening a large number of clones, but it is useful for almost every plant species. This method is especially useful when two species have very different DNA sequences. The third method reduces the amount of screening, but it involves more complicated techniques, such as F-PERT. This method is especially useful when two DNA populations possess very similar DNA sequences.

RFLP analysis

Restriction fragment length polymorphisms (RFLPs) are detected by Southern hybridisation if any of the following changes occur: base-pair change within a restriction enzyme recognition site, deletion or insertion within a restriction fragment, or chromosomal rearrangement. True hybrids should show bands inherited from both parents. Analysis of rDNA RFLPs confirmed somatic hybrids between *Brassica oleracea* and *B. campestris* (Rosen *et al.* 1988), and between *Nicotiana tabacum* and *N. rustica* (Pental *et al.* 1988). Because the coding region of rDNA is highly conserved, rDNA clones from one species can be used to detect RFLP in species even from different genera or families (Honda & Hirai 1990). By RFLP analysis with cDNA probes, somatic hybrids were confirmed between *Solanum tuberosum* (potato) and *S. brevidens* (Fish *et al.* 1988; Pehu 1989). Highly repetitive DNA may be not suitable for RFLP analysis because too many bands may be present making interpretation difficult.

A cDNA clone contains DNA sequences normally derived from single copy or low copy number DNA. When probes made from this type of clones are used in RFLP analysis for hybrid identification, some asymmetric hybrids may not be detected if a chromosome or chromosomal fragment which contains the DNA fragment is eliminated. For example, when a patatin (the major soluble protein of potato tuber) cDNA clone was used to identify somatic hybrids between *S. tuberosum* and *S. brevidens*, six plants had varying numbers of bands from the *S. brevidens* in addition to *S. tuberosum* bands and two plants contained bands apparently only of *S. brevidens* DNA. When a random cDNA clone was used, seven of these eight plants produced clear hybrid RFLP patterns (Pehu *et al.* 1989). As all these eight plants were

aneuploid, they could have lost the chromosome(s) carrying the patatin loci. This problem is less likely to occur with repetitive DNA probes.

An alternative to RFLP analysis, denaturing gradient gel electrophoresis (DGGE), has been used to identify DNA polymorphism (Lerman *et al.* 1986). Genomic DGGE analysis identifies DNA polymorphism on the basis of change in the melting properties of genomic DNA fragments. Changes as small as a single base-pair difference between two DNA fragment of equal size can be detected by this technique. Using this technique, genomic DNA polymorphism with high frequency has been identified in maize (Riedel *et al.* 1990). Theoretically, this technique could also be used to identify hybrids.

Advantages of molecular techniques in hybrid identification

Compared with isozyme techniques, molecular biology techniques have several advantages for hybrid identification. First of all, DNA is relatively stable compared to isozymes. Enzyme activity may be lost during extraction, electrophoresis, or during the colour detection process. Secondly, the expression of enzyme may be tissue specific, developmental stage specific, or affected by the environment. Nuclear DNA is present in every type of tissue, at all developmental stages. Finally, isozymes are encoded by a few loci which may be lost following chromosome elimination or deletion. So, isozyme analysis may not detect asymmetric hybrids. Repetitive DNA probes, however, can virtually avoid this problem.

4. Plastid DNA inheritance in sexual hybrids

Most angiosperm species inherit maternal plastid (pt) DNA. However, biparental ptDNA inheritance has been found in a number of species. Based upon cytological evidence, 18% species of 235 species studied are potentially capable of biparental transmission of ptDNA (Corriveau & Coleman 1988). From a more recent review (Harris & Ingram 1991), of the 398 species that have been studied, 27% show the potential for biparental ptDNA transmission. Detailed studies have been made on biparental ptDNA inheritance in genera *Pelargonium* (Tilney-Bassett & Almouslem 1989), *Medicago* (Lee *et al.* 1988, Smith 1989a) and *Oenothera* (Chiu *et al.* 1988). Furthermore, strong paternal ptDNA inheritance has been reported in *Medicago sativa*

(Schumann & Hancock 1989, Masoud *et al.* 1990) and in the genus *Daucus* (Boblenz *et al.* 1990).

In contrast to the angiosperms, ptDNA inheritance in the gymnosperms is mostly paternal. High levels of paternal ptDNA inheritance have been documented in *Pinus* (Wagner *et al.* 1989), *Larix* (Szmidt *et al.* 1987), *Pseudotsuga* (Neale *et al.* 1986) and *Picea* (Stine & Keathley 1990, Sutton *et al.* 1991).

Three methods have been used to study ptDNA inheritance. Genetic analysis of plastid mutant, the earliest used method, uses phenotypically recognisable plastid characters, such as green against white (Kirk & Tilney-Bassett 1978, Tilney-Bassett & Almouslem 1989). This method may be not suitable for many plant species because a phenotypical distinguishable plastid mutant may be not available. The second method is to detect ptDNA in the generative cell and/or pollen tube with epifluorescence microscopy after DNA is stained with 4',6-diamidino-2-phenyl-indole (DAPI). This method can screen a large number of plants rapidly for detecting potential biparental ptDNA inheritance (Corriveau & Coleman 1988). Plant species are more likely to inherit biparental ptDNA if ptDNA is detected in generative cells and/or pollen tube (Corriveau & Coleman 1988, Harris & Ingram 1991). The third method is ptDNA restriction analysis. This method could be used for a wider range of species than the first method and much more reliable than the second method. DNA restriction analysis allows a more meaningful estimation of the frequency of plastid transmission than does genetic analysis of chlorophyll deficient mutants, which may be transmitted at a competitive disadvantage. In *Pelargonium*, green plastids were transmitted at a higher rate than were mutant plastids (Tilney-Bassett & Birky 1981, Tilney-Bassett & Abdel-Wahab 1982).

Plastid DNA restriction analysis can be done either by directly comparing ptDNA bands in an ethidium bromide stained gel (Metzlaff *et al.* 1981, Szmidt *et al.* 1987, Horlow *et al.* 1990) or by Southern hybridisation (Lee *et al.* 1988, Stine & Keathley 1990, Mejnartowicz 1991). The former analysis requires purified ptDNA. Purification of ptDNA may be difficult from some plant materials, such as albino leaves. The second method requires cloned ptDNA to be used as probes but total cellular DNA can be used in the Southern blots. Plastid DNA clones from different species may be used as probes (Schumann & Hancock 1989) because ptDNA sequences are highly conserved in higher plants (Palmer 1987).

CHAPTER 4. A study of somatic chromosomes in the genus *Zantedeschia*

1. Introduction

Chromosome studies have contributed a great deal to the interpretation of the phylogenetic relationships between different taxonomic groups (Hsiao *et al.* 1986). These relationships can give clues to the selection of compatible parents. Chromosome analyses can be used to identify hybrids (Pittarelli & Stavely 1975, Mohapatra & Bajaj 1987, Przywara *et al.* 1989), detect chromosome elimination (Pijnacker *et al.* 1987) and mutations in hybrids (Iwai *et al.* 1985, Shintaku *et al.* 1988).

Chromosomes of *Z. aethiopica* and five species in section II of the genus *Zantedeschia* were studied by Earl (1957). She reported that all the species examined had 32 chromosomes with centromeres that appeared to be at a median to sub-median location. No other chromosomal details were observed because of the small size of the chromosomes and no photographic evidence was provided. Chromosomes of a new species *Z. odorata* have not previously been described. The objectives in this study were to determine the chromosome number of *Z. odorata* and prepare karyotypes of most species in the genus. It was hoped that these cytogenetical data would be useful in the selection of compatible parents for the interspecific hybridisation program and later to assist in the identification of hybrids.

2. Material and methods

Chromosome preparation. Chromosome preparations were made of five species and two cultivars. These species and cultivars with their code and descriptions are listed in Table 2. Rapidly growing root-tips were obtained by planting rhizomes or tubers in potting mix in a greenhouse. Root-tips were pretreated in a saturated solution of para-dichlorobenzene for four hours and fixed in methanol : glacial acetic acid 3:1 overnight and stored in 70% ethanol. Two staining methods were used. Initially Feulgen was used but improved staining was subsequently achieved with Giemsa. 1. Fixed root-tips of EL, PE, RE, CHR, MR, and CHI were hydrolysed in 1N HCl at 60 °C

for ten minutes and stained with Feulgen stain for 60 minutes. Stained root-tips were washed in distilled water for several times. Root meristems were dissected out, macerated on microscope slides in 2% acetocarmine, and squashed under coverslips in the usual manner after heating each slide on a hot block for approximately one minute at 80 °C. Coverslips were removed after slides were chilled in liquid air. **2.** Fixed root-tips of OD, AE and CHI were macerated on microscope slides in 45% acetic acid, squashed under coverslips in the usual manner after heating each slide on a hot block for approximately one minute at 80 °C. After the coverslips were removed by chilling in liquid air, the slides were air dried overnight. The slides were stained in 5% Giemsa in phosphate buffer (pH 6.8) for two hours and then rinsed in distilled water. Chromosomes were observed and photographed using a Zeiss Photomicroscope III.

Table 2. *Zantedeschia* taxa used in chromosome study

Taxa	Code	Description
<i>Z. aethiopica</i> (L.) Spreng	AE	evergreen, Letty (1973)
<i>Z. aethiopica</i> 'Childsiana'	CHI	evergreen, floriferous dwarf form, Bailey & Bailey (1976)
<i>Z. odorata</i> P. L. Perry	OD	summer dormant, Perry (1989)
<i>Z. elliottiana</i> (Watson) Engl.	EL	winter dormant Letty (1973)
<i>Z. pentlandii</i> (Watson) Wittm.	PE	winter dormant Letty (1973)
<i>Z. rehmannii</i> Engl.	RE	winter dormant Letty (1973)
<i>Z.</i> 'Chromatella'	CHR	winter dormant hybrid related to <i>Z. albomaculata</i> .
<i>Z.</i> 'Majestic Red'	MR	winter dormant hybrid, parentage unknown.

Karyotype analysis. The length of short and long arms of each chromosomes was measured. Chromosomes were paired on the basis of chromosome length and arm ratio. Chromosome relative length, arm ratio and total form percentage of short arms (TF%) were calculated with the formulae below.

$$\text{relative length} = \frac{\text{length of a chromosome}}{\text{total complement length}} \times 100$$

$$\text{arm ratio (r)} = \frac{\text{length of long arm}}{\text{length of short arm}}$$

$$\text{TF\%} = \frac{\text{total length of short arms}}{\text{total complement length}} \times 100 \quad (\text{Hamal \& Koul 1988})$$

The descriptions of centromeric position followed the standard nomenclature of Levan *et al.* (1964), which is summarised in Table 3. Karyotype asymmetry was evaluated against Stebbins’ chart (1971) (Table 4). Asymmetry is determined by the centromeric locations and uniformity of the chromosome sizes. Twelve types of karyotype are classified in this chart. Asymmetry increases from type 1 to type 4 according to the proportion of chromosomes with an arm ratio higher than 2.0. Asymmetry increases from type A to type C according to the size ratio between the largest chromosome and smallest chromosome.

Table 3. Nomenclature of centromeric position (Levan *et al.* 1964)

centromeric position	chromosome designation		arm ratio
median sensu stricto	M	Metacentric	1.0
median region	m		1.7
submedian	sm	Submetacentric	3.0
subterminal	st	Subtelocentric	7.0
terminal region	t	Acrocentric	
terminal sensu stricto	T	Telocentric	∞

Table 4. A classification of karyotypes according to their degree of asymmetry (Stebbins 1971)

largest : smallest	proportion of chromosomes with arm ratio > 2:1			
	0.0	0.01-0.5	0.51-0.99	1.0
< 2:1	1A	2A	3A	4A
2:1-4:1	1B	2B	3B	4B
> 4:1	1C	2C	3C	4C

3. Results.

Chromosome number. Chromosome counts were made for more than 50 mitoses of root-tip cells of the new species *Z. odorata*. The chromosome number of this species was determined as 32 (Fig. 1.2) for the first time. All other *Zantedeschia* species and hybrids were also found to have 32 chromosomes in a diploid cell (Fig. 1). This result confirmed previous observations (Earl 1957). A few cells with tetraploid and aneuploid chromosome numbers were observed. The tetraploid cells may be caused by an endomitosis mechanism and the aneuploids may be caused by unequal separation. In one anaphase cell of CHI, 27 chromosomes at one pole and 37 chromosomes at the other pole were observed (Fig. 3). This observation is open to interpretation. It could indicate non-segregation of some chromosomes as is seen in some other plant species or it may merely reflect the direction of squashing. Nevertheless the author feels the configuration should be noted as a point of interest. Bianco *et al.* (1991) observed tetraploid, hexaploid and octoploid cells in the ovary wall of a diploid plant of *Ophrys*. The tetraploid and octoploid cells probably resulted from endomitosis while the hexaploid cells could arise through fusion between nuclei.

Karyotype of *Z. aethiopica* 'Childsiana' (CHI). The somatic complement comprised two pairs of metacentric chromosomes (m), two pairs of submetacentric chromosomes (sm), 11 pairs of subtelocentric chromosomes (st) and one pair of acrocentric chromosomes (Fig. 2.1, Table 5). The average arm ratio and relative length, as well as the SE, of each pair of chromosomes are listed in Table 5. The chromosomes were small, under 3 μm long (Fig. 1.1). The ratio between the largest and smallest chromosomes was 1.77. Arm ratios of 87.5% of the chromosomes were higher than

2.0. According to Stebbins' classification (Table 4), the karyotype of this species was the type 3A. The TF% of this species was 25.77 ± 0.26 . The chromosomes of *Z. aethiopica* (AE) were also observed and could not be distinguished from those of *Z. aethiopica* 'Childsiana' (Fig. 1.1a).

Table 5. Chromosome relative length and arm ratio of *Z. aethiopica* 'Childsiana' *

chromosome	relative length (mean \pm SE)	arm ratio (mean \pm SE)
m1	6.72 ± 0.12	1.11 ± 0.03
m2	6.37 ± 0.08	1.03 ± 0.01
sm1	7.20 ± 0.10	2.00 ± 0.09
sm2	6.41 ± 0.13	2.13 ± 0.07
st1	7.96 ± 0.05	3.77 ± 0.18
st2	7.31 ± 0.04	3.80 ± 0.14
st3	6.85 ± 0.12	3.55 ± 0.18
st4	6.61 ± 0.06	3.51 ± 0.16
st5	6.24 ± 0.10	3.53 ± 0.20
st6	5.91 ± 0.10	3.52 ± 0.22
st7	5.72 ± 0.07	3.96 ± 0.28
st8	5.50 ± 0.07	3.80 ± 0.26
st9	5.28 ± 0.07	3.84 ± 0.09
st10	4.89 ± 0.09	4.13 ± 0.30
st11	4.52 ± 0.08	4.66 ± 0.26
t1	6.49 ± 0.16	9.19 ± 0.59

* The data was obtained from ten metaphases. Chromosomes are numbered according to their relative length within a group, from the longest to the smallest.

In CHI, one pair of acrocentric chromosomes was distinct from all other chromosomes. The two pairs of metacentric chromosomes were distinguishable from other chromosome groups and usually were distinguishable from each other. Similarly, the submetacentric chromosomes were readily distinguishable from the other groups and within themselves on the basis of centromere position. The subtelocentric

chromosomes were a distinctive group but they were not all distinguishable within the group. They showed a gradient of decreasing length among the eleven pairs.

Karyotype of *Z. odorata* (OD). This species had four pairs of metacentric chromosomes (m), five pairs of submetacentric chromosomes (sm), six pairs of subtelocentric chromosomes (st), and one pair of acrocentric chromosomes (Fig. 2.2, Table 6). The arm ratio and the relative length of each pair of chromosomes are summarised in Table 6. Chromosomes were small, similar to those of CHI (Fig. 1.2). The karyotype was type 3B as 75% of chromosomes had an arm ratio higher than 2.0 and the ratio between the largest and smallest chromosomes was 2.32. The TF% was 29.59 ± 0.25 .

Table 6. Chromosome relative length and arm ratio of *Z. odorata* *

chromosome	relative length (mean \pm SE)	arm ratio (mean \pm SE)
m1	7.14 ± 0.18	1.04 ± 0.01
m2	6.61 ± 0.12	1.05 ± 0.01
m3	6.13 ± 0.11	1.03 ± 0.01
m4	4.27 ± 0.15	1.11 ± 0.03
sm1	9.02 ± 0.25	2.46 ± 0.10
sm2	7.81 ± 0.16	2.26 ± 0.08
sm3	7.14 ± 0.10	2.47 ± 0.08
sm4	6.09 ± 0.23	2.50 ± 0.15
sm5	5.09 ± 0.21	2.61 ± 0.15
st1	7.86 ± 0.24	3.71 ± 0.12
st2	6.84 ± 0.13	4.17 ± 0.37
st3	5.56 ± 0.18	4.32 ± 0.23
st4	5.17 ± 0.20	4.00 ± 0.21
st5	4.57 ± 0.11	4.02 ± 0.14
st6	3.99 ± 0.13	4.75 ± 0.25
t1	6.70 ± 0.11	9.46 ± 0.54

* As described in Table 5.

One pair of acrocentric chromosomes and a pair of small metacentric chromosomes were distinct in the complement. The acrocentric pair appeared to be the same in CHI and OD. The karyotypes of CHI and OD were similar except for two differences: OD had two more metacentric chromosome pairs and had a higher ratio between the largest and smallest chromosomes than CHI. Although the number of submetacentric chromosome pairs was different, it was not always obvious as some submetacentric and some subtelocentric chromosomes could be mis-classified.

Karyotype of *Z. elliottiana* (EL). This species had 11 pairs of chromosomes with centromeres at the median region and five pairs of chromosomes with submedian centromeres. Of the 11 metacentric pairs, seven pairs had centromeres close to the median point (Fig. 2.3), like those metacentric chromosomes in CHI and OD. However, the remaining four metacentric pairs had centromeres away from the median point although their arm ratios are less than 1.7 (Table 7). Chromosomes in this species were also small, similar to that of CHI and OD. The arm ratio and relative length of each pair of chromosomes are listed in Table 7. The karyotype fell in the type 2A with 12.5% chromosomes having an arm ratio larger than 2.00 and with a ratio of 1.58 between the largest and smallest chromosomes. The TF% was 40.91 ± 0.43 . No chromosome was distinct in this species. This karyotype was very different from those of CHI and OD.

Karyotype of other species and cultivars in section II. *Z. pentlandii* and *Z. rehmannii* had ten pairs of metacentric chromosomes (m) and six pairs of submetacentric chromosomes (sm). *Z. 'Chromatella'* had 11 pairs of metacentric chromosomes and five pairs of submetacentric chromosomes. *Z. 'Majestic Red'* had nine pairs of metacentric chromosomes and seven pairs of submetacentric chromosomes (Table 8). The karyotypes of these species and cultivars (Fig. 2.4 to Fig. 2.7) were very similar to that of *Z. elliottiana* (Fig. 2.3). Chromosomes in these complements were either metacentric or submetacentric. The metacentric chromosomes could be readily divided into two subgroups. In one group, the centromeres were very close to the median point but in the other group the centromeres were away from the median point although the arm ratios were less than 1.7. The numbers of metacentric chromosome pairs (9 to 11) and submetacentric chromosome pairs (5 to 7) were very close in different species (Table 8). This slight variation may be accounted for by the misplacement of one metacentric or

submetacentric pair. The TF% of these five taxa in section II were from 40.52 to 41.94 and were not significantly different ($p=0.05$) from one another. The ratios between the largest and smallest chromosomes were close to 2 (Table 8). These karyotypes were classified to the type 2A. Chromosome sizes were similar among these species or cultivars and smaller than 3 μm long (Fig 1.3 to Fig. 1.7). These karyotypes, therefore, can not be easily distinguished from each other. To distinguish these karyotypes, more advanced cytological techniques are required for observing more chromosomal details.

Table 7. Chromosome relative length and arm ratio of *Z. elliotiana* *

chromosome	relative length (mean \pm SE)	arm ratio (mean \pm SE)
m1	7.84 \pm 0.11	1.16 \pm 0.04
m2	7.13 \pm 0.11	1.15 \pm 0.05
m3	6.62 \pm 0.10	1.12 \pm 0.03
m4	6.24 \pm 0.16	1.15 \pm 0.04
m5	5.92 \pm 0.15	1.15 \pm 0.04
m6	5.61 \pm 0.15	1.22 \pm 0.05
m7	5.35 \pm 0.23	1.20 \pm 0.04
m8	7.30 \pm 0.26	1.58 \pm 0.04
m9	6.30 \pm 0.26	1.53 \pm 0.04
m10	5.93 \pm 0.23	1.49 \pm 0.04
m11	5.31 \pm 0.25	1.55 \pm 0.04
sm1	7.18 \pm 0.23	2.04 \pm 0.07
sm2	6.43 \pm 0.30	2.00 \pm 0.05
sm3	5.95 \pm 0.17	1.94 \pm 0.09
sm4	5.48 \pm 0.15	1.95 \pm 0.05
sm5	4.96 \pm 0.07	2.04 \pm 0.09

* As described in Table 5.

Table 8. A summary of TF%, ratio between the largest and smallest chromosomes and types of chromosome from five species and two cultivars in *Zantedeschia* *

species or cultivar	TF% (mean ** \pm SE)	Largest/smallest (mean \pm SE)	No. of chromosome pairs			
			m	sm	st	t
CHI	25.77 ^a \pm 0.26	1.77 \pm 0.12	2	2	11	1
OD	29.59 ^b \pm 0.25	2.32 \pm 0.25	4	5	6	1
EL	40.91 ^c \pm 0.43	1.58 \pm 0.12	11	5	0	0
PE	40.52 ^c \pm 0.60	1.98 \pm 0.04	10	6	0	0
RE	40.94 ^c \pm 0.60	1.86 \pm 0.08	10	6	0	0
MR	41.90 ^c \pm 0.60	1.76 \pm 0.05	9	7	0	0
CHR	41.94 ^c \pm 0.46	1.91 \pm 0.07	11	5	0	0

* Data were collected from ten cells of CHI, OD and EL, but from five cells of the remaining plant material.

** Means followed by the same letter are not significantly different ($p=0.05$) as indicated by ANOVA.

Fig. 1. Metaphase spreads of *Zantedeschia* species and cultivars

(1): *Z. aethiopica* 'Childsiana'

(1a): *Z. aethiopica*

(2): *Z. odorata*

(3): *Z. elliottiana*

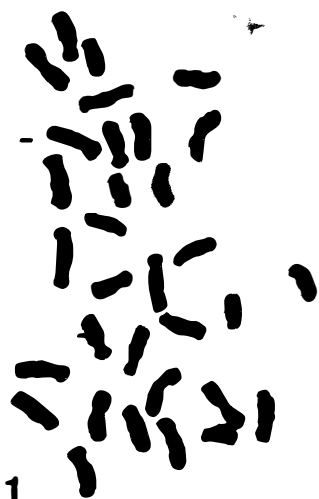
(4): *Z. pentlandii*

(5): *Z. rehmannii*

(6): *Z.* 'Chromatella'

(7): *Z.* 'Majestic Red'

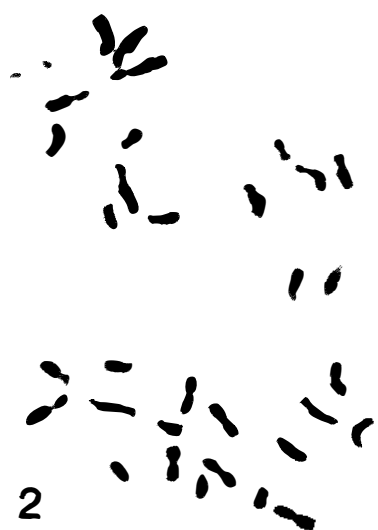
Chromosomes in (1), (1a) and (2) were stained in Giemsa, others were stained in Feulgen (see Material and method section). Bar: 5 μ m.



1



1a



2



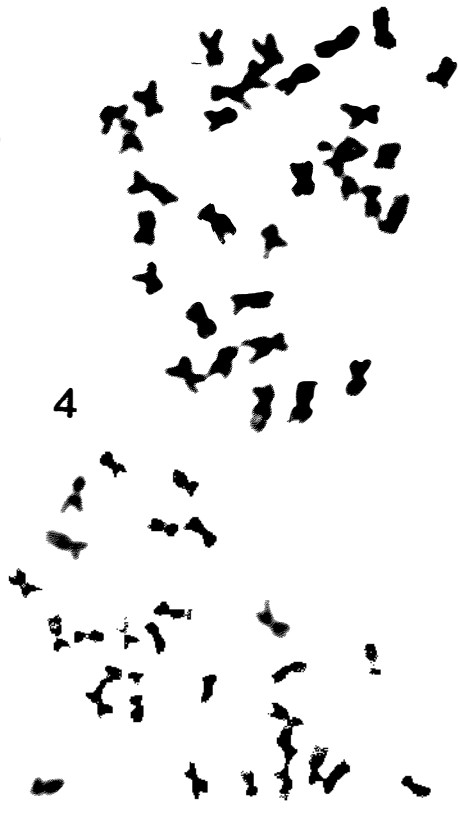
3



5



6



4



7

11

Fig. 2. Karyotypes of *Zantedeschia* species and cultivars

(1): *Z. aethiopica* 'Childsiana'

(2): *Z. odorata*

(3): *Z. elliottiana*

(4): *Z. pentlandii*

(5): *Z. rehmannii*

(6): *Z.* 'Chromatella'

(7): *Z.* 'Majestic Red'

These karyotypes were prepared from the cells in Fig. 1.

1 1111 2222 3333 4444 5555 6666 7777 8888 9999 0000

2 1111 22 3333 4444 5555 6666 7777 8888 9999 0000

3 1111222233334444555566667777888899990000

4 1122334455667788990011223344556677889900

5 1122334455667788990011223344556677889900

6 1122334455667788990011223344556677889900

7 1122334455667788990011223344556677889900

5 μ m

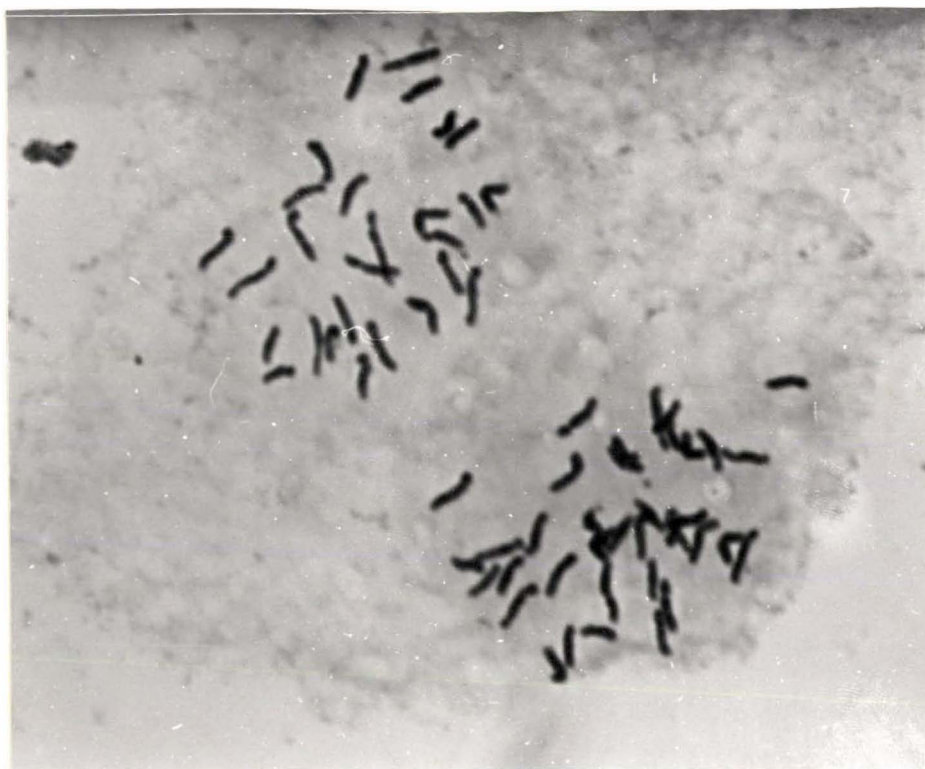


Fig. 3. An anaphase mitotic cell of *Z. aethiopica* 'Childsiana'

There are 27 chromosomes in one pole and 37 chromosomes in another pole. Chromosomes were stained in Feulgen.

4. Discussion

This study describes the karyotype of *Z. odorata* (OD) for the first time and gives comparable but more detailed karyotype data for other species of the genus *Zantedeschia*. The chromosome number, 32 for each species, is the same as determined previously (Earl 1957). Earl's publication includes only drawings but no photographs. She reported that the centromeres appeared to be in a "medium to submedian location" for *Z. aethiopica* and five species in section II of *Zantedeschia*. A detailed analysis of the chromosomes of section I, section II and of the new species OD in the current study shows important differences in the karyotypes not noted by Earl. *Z. aethiopica* has mostly subtelocentric chromosomes whereas species and cultivars in section II have all chromosomes with a median or submedian centromeric location (Table 8). OD has more chromosomes with a median or submedian centromeric location than *Z. aethiopica*, but less than species in section II.

The karyotypes of section I and section II are quite distinct and there is very little variation within section II. This suggests that the species in section II are closely related to each other and the two sections are clearly separated. This conclusion agrees not only with Letty's (1973) classification based on morphological characters but also with the results of interspecific hybridisation. Different species within section II can cross with each other (Traub 1949, New & Paris 1967, Chi 1990). Only albino hybrids were produced from the crosses between the two sections, even though different genotypes from both sections were used in the crosses and embryo culture techniques were used for embryo rescue (Chi 1990, Chapter 6).

In the plant kingdom, the predominant trend is an increase in asymmetry as a species evolves, although reversals of this trend occur periodically (Stebbins 1971). Using Stebbins' karyotype classification, the karyotype (3B) of OD has the greatest asymmetry followed by CHI (3A) and then section II (2A). Based on morphological characters, OD falls in between the two sections (Perry 1989). OD can hybridise with *Z. aethiopica* and improve crossability to section II genotypes compared with crosses between *Z. aethiopica* and section II (Chapter 7). This result confirms that OD is intermediate between the two sections but is more closely related to *Z. aethiopica* than to species in section II. Stebbins' system of karyotype classification may not be suitable in this genus for phylogenetical analysis. On one hand, the difference in the centromeric positions between OD and CHI is ignored by classifying 87.5% (for CHI) and 75% (for OD) of chromosomes with arm ratio larger than 2.0 into the same type.

On the other hand, it puts too much emphasis on the size ratio between the largest and smallest chromosomes by using the standard of 2.0 to divide 1.76 (for CHI) and 2.26 (for OD) into two different types.

An asymmetric analysis based on the TF% appears to give a better indication of phylogenetic relationships. The TF% of CHI and OD are 25.77 and 29.59 respectively. An average TF% of species and cultivars in section II is 41.24. The TF% is not significantly different ($p=0.05$) within section II, but the TF% is significantly different among CHI, OD and section II (Table 8). The TF% increases from CHI (section I) to OD to section II. Thus OD falls in between the two sections. Since the TF% of OD is closer to the TF% of CHI, OD would appear to be more closely related to section I. This interpretation is compatible with the morphological characters and interspecific crossabilities.

Centromeric position can be altered by either pericentric inversion, unequal translocation or chromosome "fusion" between two acrocentrics or telocentrics (Stebbins 1971). A consequence of fusion is a reduction in chromosome number and an increase in chromosome sizes. Unequal translocation changes chromosome sizes as it alters the centromeric positions. Pericentric inversions change the centromeric position only. The chromosome numbers are the same in all *Zantedeschia* species. So chromosome fusion does not appear to be a factor in the evolution of the genus. One of the major differences between the karyotypes of CHI and OD is the size ratio between the largest chromosome and smallest chromosome. This could be due to unequal translocation which changed centromeric locations. Chromosome sizes are very similar in all species (Fig. 1). Thus the centromeric position alterations may be predominantly caused by pericentric inversions. Earl (1957) suggested that speciation in the genus has proceeded chiefly by gene mutation based on the similar chromosome complements, the ease of hybridisation within the summer flowering section and behaviour of the meiotic chromosomes in the hybrids. The current study agrees with this suggestion within section II only. Chromosome alterations, such as pericentric inversions and unequal translocations, may have played an important role in the speciation of the genus *Zantedeschia*.

This study has demonstrated the clear karyotype difference between *Z. aethiopica* and species in section II. However, the karyotypes for different species in section II are very similar. Therefore, an attempt to select a species in section II most closely related to *Z. aethiopica* for hybridisation is not possible from these data. The new species,

OD, has a karyotype distinguishable from the karyotypes of species in the two previously described sections. Karyotype asymmetry based on the TF% has located OD between *Z. aethiopica* and section II. This result has encouraged us to use OD as a bridge species for gene transfer between the two sections.

Successful hybridisation between two species could give rise to a hybrid offspring with a distinctive karyotype of its own. This feature may be used for hybrid identification. But karyotype preparation is time consuming. It may not be suitable to screen a large number of plants. If distinctive chromosomes, such as satellite chromosomes and extremely large or small chromosomes, are present in one species but not in another, hybrid identification would be much easier by looking for these specific chromosomes. Unfortunately, such distinctive chromosomes are not present in *Zantedeschia*.

Chromosome banding has been used to identify plant chromosomes. But banding small plant chromosomes is normally difficult. Imaging methods have been used to identify small rice chromosomes at mitotic pro-metaphase (Fukui and Iijima 1991, Iijima *et al.* 1991). At pro-metaphase, rice chromosomes have uneven stainability which is due to the differential compactness of the chromatin fibres along the chromosome. This uneven stainability is the most important character in the image analysis. In the current study, a C-banding technique with denaturation in saturated Ba(OH)_2 and renaturation in 2 X SSC at 60 °C (Zhu *et al.* 1982) was tried but was not successful. The uneven staining of pro-metaphase chromosomes of *Zantedeschia* was observed. Image analysis of pro-metaphase chromosomes may eventually be useful for identifying *Zantedeschia* chromosomes.

CHAPTER 5. Production and identification of tetraploid and triploid plants

1. Introduction

Polyploid plants have been widely used in plant breeding programs in order to: 1) directly generate new cultivars, 2) restore the fertility of intergeneric or interspecific hybrids, 3) overcome crossing incompatibility. Since naturally evolved polyploid strains are usually unavailable, it is usually necessary to induce polyploidy.

Conventional polyploid inducing methods, such as colchicine treatment of seedlings *in vivo*, produce polyploids with low efficiency and there is also a high frequency of chimerism (Arisumi 1964, Pryor & Frazier 1968, Semeniuk & Arisumi 1968, Ackerman & Dermen 1972, Schifino & Fernandes 1987, Griesbach 1990). It is often difficult to treat a large number of seedlings *in vivo* and many of the treated seedlings may die. The colchicine treatment may only affect a portion of the cells in a meristem and plants generated from these meristems may be either periclinal chimeras or mericlinal chimeras depending on the location of colchicine-affected cells. In the case of mericlinal chimeras, sectors with different ploidy levels often separate in vegetatively propagated plants. Periclinal chimeras are usually more stable. If the polyploid cells are located in the L₂ layer of the meristem, they will be transmitted to sexually formed progeny. The process of chimerism separation is often slow and complicated.

In vitro plant tissue culture has been used to produce polyploids since 1966 (Murashige & Nakano). Murashige and Nakano obtained tetraploid tobacco plants from suspension cells derived from a pith of diploid *Nicotiana tabacum*. Later, a high percentage (50%) of polyploids with a few mixoploids (4%) were produced from colchicine-treated cell suspension cultures of sugarcane (Heinz & Mee 1970). More recently, chromosome doubling has been achieved from various plant species by *in vitro* colchicine treatment of different types of tissue: suspension cells (Chavadej & Becker 1984, Dolezel & Binarova 1989), callus (Chen & Goeden-Kallemeyn 1979, Orton & Steidl 1980), somatic embryos (Gmitter *et al.* 1991), axillary meristems (Lyrene & Perry 1982, Anderson *et al.* 1991), and anthers (Barnabas *et al.* 1991). The *in vitro* methods can avoid or reduce chimeras because plants are generated from a single cell or a few cells in the case of cell and callus culture. Mericlinal chimeras may

segregate during the shoot multiplication stage in the case of colchicine treatment of multiple shoots. These methods also gave a higher efficiency of polyploid production because a large number of cells or multiple shoots can be easily treated in a culture container. Thus, even though many of the cells or shoots die after the treatment, surviving cells or shoots can be multiplied rapidly to obtain a number of polyploid plants.

A method to identify polyploids is essential after colchicine treatment because many of the regenerated plants may be diploids. Chromosome number is the most reliable indicator for ploidy level, but chromosome counting is time consuming. Many alternative indicators have been used for identifying plant ploidy level, such as: stomatal size (Speckmann *et al.* 1965, Tan & Dunn 1973, Borrino & Powell 1988, Przywara *et al.* 1988), chloroplast number in stomatal guard cells (Bingham 1968, Chaudhari & Barrow 1975, Kamo & Griesbach 1989), pollen size (Tan & Dunn 1973), and nuclear DNA content (Dolezel & Binarova 1989, Kamo & Griesbach 1989). Of these indicators, stomatal size has been most widely used because the measurement is simple and does not require expensive instruments.

In *Zantedeschia*, all natural species and hybrids are diploid and no induced polyploid plant has been reported from our knowledge. The usefulness of polyploidy in calla lily improvement is unknown. In this study, colchicine treatment of multiple shoots *in vitro* was used to produce tetraploid plants from a number of summer-flowering cultivars. Colchicine treatment of embryos and germinating seeds was applied to produce tetraploid plants from *Z. aethiopica*. Putative tetraploid plants selected by stomatal measurements were verified with chromosome counting. In addition, embryo culture was used to recover triploid plants from crosses between diploid and tetraploid plants.

2. Material and methods

2.1. Plant material. Nine New Zealand bred summer-flowering *Zantedeschia* cultivars: 'Black magic' (BM), 'Galaxy' (G), 'Golden Sun' (GS), 'Pacific Pink' (PPK), 'Purple Star' (PST), 'Red Beauty' (RB), 'Red Emperor' (RE), 'Red Gold' (RG), 'Rose Queen' (RQ) were used in the first experiment for *in vitro* colchicine treatment. The parentage of these hybrid cultivars is unknown. *Z. pentlandii* (PE), *Z. elliottiana* (EL) and *Z. rehmannii* (RE) were used in the second experiment for *in vitro* colchicine

treatment. Embryos of *Z. aethiopica* 'Childsiana' (CHI) and a summer-flowering cultivar 'Chromatella' (CHR) were used for *in vitro* colchicine treatment. Germinating seeds of CHI and CHR were treated with colchicine. Tetraploid GS and tetraploid RE were crossed with EL and summer-flowering cultivars coded 'C', 'D' and 'WP', in order to produce triploid plants.

2.2. Surface sterilisation. Seeds, tubers and ovaries were sterilised in 0.6% sodium hypochlorite (15% of a commercial bleach containing 4% active ingredient) for 15 minutes with shaking. A non-ionic detergent (multifilm X77) was added to 0.05% to improve the wetting of the explants. After the hypochlorite treatment, tubers and ovaries were briefly rinsed in 95% ethanol and air dried. Dry seeds were soaked in water for up to four days to soften the endosperm before hypochlorite treatment. They were then rinsed three times with sterile water before embryo dissection.

2.3. Medium preparation. The composition of media consisted of Murashige and Skoog (1962) minerals with the following additives: myoinositol 100 mg/l, thiamine-HCl 0.4 mg/l, sucrose 30 g/l, Davis agar 7 g/l and benzyladenine (BA) at various concentrations. The pH of media was adjusted to 5.8 before the agar was added. Media were autoclaved at 1.1 kg/cm² (121 °C) for 15 minutes. If colchicine was used, it was added into the medium prior to the autoclaving.

2.4. Colchicine treatment of multiplying shoots *In vitro*. The method for *in vitro* shoot multiplication from bud explants was described by Cohen(1981). Dissected buds were initially cultured on medium containing 3 mg/l BA (first experiment) or 0.3 mg/l BA (second experiment). After the establishment of rapidly multiplying cultures, shoots were transferred to a medium containing 3 mg/l BA and 0.05%(w/v) colchicine for 1, 2 or 4 days. Similar cultures without colchicine treatment were used as controls. After the colchicine treatment, shoots were transferred to medium with 3 mg/l BA for one (first experiment) or two (second experiment) subcultures to multiply the surviving tissue. They were then transferred to a medium with 0.1 mg/l BA for shoot elongation. Elongated shoots were transferred into jars containing a medium with 0.1 mg/l BA for rooting. Small multiplying shoots were subcultured on fresh plates containing a medium with 0.3 mg/l BA. Rooted shoots were transplanted into potting mix in a greenhouse.

2.5. Colchicine treatment of embryos *in vitro*. Embryos were dissected out from sterilised seeds under aseptic condition and cultured on medium with 0.3 mg/l BA for

four days to initiate embryo growth. Growing embryos were transferred to a medium with 0.05% colchicine and 0.3 mg/l BA for one or two days. Embryos were then transferred back to colchicine-free medium.

2.6. Colchicine treatment of germinating seeds. Seeds were germinated on wet filter paper in petri-dishes at 20 °C. Germinating seeds (after five weeks in germination condition, see Fig. 11) were immersed in 0.05% colchicine solution for 24 hours and washed several times in water. The seeds were then sown in potting mix in a greenhouse.

2.7. Stomatal size measurement. The size of stomata was measured on leaf impressions prepared via the following manner. A small area (about 1 cm²) on the upper epidermis of leaves was smeared with PVA (white glue). After the glue dried, the epidermis of this area was peeled off using a strip of sellotape. The tape was stuck onto a microscope slide. A Reichert Visopan microscope with a viewing screen was used at 63X magnification. On the viewing screen 1 mm = 1.25 µm at this magnification.

2.8. Selecting putative tetraploid plants based on stomatal size. Stomatal measurements (Section 2.7) were made from both colchicine-treated and control plants between two and three months after the plants were transplanted into a greenhouse. The length of ten stomata of one sample was averaged to obtain the stomatal size for each plant. For each cultivar, the stomatal size of 7 to 15 control plants was used to calculate the control mean and standard deviation (SD). The stomatal size of each plant from colchicine treatments was then compared with the control mean. If it were more than 2X SD greater than the control mean, the plant were selected as a putative tetraploid plant.

2.9. Investigation of stomatal size in mature plants. Stomatal measurements (Section 2.7) were made on 'Black Magic' plants at the flowering time of the third replanting. Five shoots were chosen from each of four diploid plants and from each of four tetraploid plants. Two samples were taken from each leaf, on each side of the mid-rib, and two leaves for each shoot. Twenty stomata were measured from each sample. The variation between ploidy levels, between plants within ploidy level, between shoots within plant, between leaves within shoot and between samples within leaf were analysed using an ANOVA program for nested design. The length of 20 stomata was averaged to obtain the stomatal size for each sample. The stomatal size

of 80 diploid samples was used to calculate the control mean and SD. The stomatal size of each tetraploid sample was then compared with the control mean to see whether the stomatal size of every tetraploid sample was more than 2X SD greater than the control mean. After pooling the four samples of a single shoot, the length of 80 stomata was averaged to obtain the stomatal size for each shoot. The stomatal size of the 20 diploid shoots was then used to calculate the control mean and SD. The stomatal size of each tetraploid shoot was then compared with the control mean to see whether the stomatal size of every tetraploid shoot was more than 2X SD greater than the control mean.

2.10. Leaf thickness measurement. Four leaf discs, 2 cm² each, were collected from each of two leaves from each plants. Leaf discs were taken from mature leaves of flowering plants using a leaf punch. The fresh weight of eight leaf discs from a single plant was obtained and the thickness of each disc was measured with a digital micrometer. The means of specific leaf weight and leaf thickness of diploid plants was compared with the means of tetraploid plants of the same cultivar using the two sample T test routine of Minitab.

2.11. Chromosome counting. Rapidly growing root-tips were pre-treated in a saturated solution of para-dichlorobenzene for 4-5 hours and fixed overnight in methanol : glacial acetic acid 3:1 (Zhu *et al.* 1982) and stored in 70% ethanol. Fixed root-tips were hydrolysed in 1N HCl at 60 °C for 10 minutes and stained with Feulgen stain for 60 minutes. Stained root-tips were washed several times in distilled water. Root meristems were dissected out, macerated on microscope slides in 2% acetocarmine, and squashed in the usual manner after heating each slide on a hot block for approximately minute at 80 °C. Coverslips were removed after the slides were chilled in liquid air. Chromosomes were observed and photographed using a Zeiss Photomicroscope III. Two to four roots were examined from each plant. The number of cells examined varied according to the mitotic index.

2.12. Producing triploid plants. Pollen from tetraploid 'Golden Sun' and tetraploid 'Red Emperor' was used to pollinate diploid plants of *Z. elliottiana* and summer-flowering hybrids, 'C', 'D' and 'WP'. Eight weeks after pollination ovaries were harvested. Sterilised ovaries (Section 2.2) were dissected to observe the development of embryo and endosperm and to provide material for *in vitro* culture. Embryos embedded within endosperm were chosen as initial culture material because the

embryos were too small to be separated from the endosperm. All explants were cultured on a medium with 0.3 mg/l BA.

3. Results

3.1. Production of tetraploid plants from summer-flowering cultivars by colchicine treatment of multiplying shoots *in vitro*

Colchicine treatment and plants generation

Cultures of nine hybrid cultivars were available in Dr. Cohen's laboratory, HortResearch, Palmerston North. Some of these cultures had been treated with colchicine one month earlier. The author of the current thesis continued the work by subculturing these cultures and later selecting for tetraploids. After colchicine treatment many shoots did not grow and some died. Surviving shoots multiplied on a medium with 3.0 mg/l BA. On a medium containing 0.1 mg/l BA, shoot multiplication slowed down, shoot elongation occurred and roots formed. Rooted plantlets were first transplanted into a greenhouse after the second subculture on a medium with 0.1 mg/l BA. Two further subcultures were conducted in order to obtain enough plants. Control plantlets were also transplanted for each hybrid. Stomatal length measurements were made on all surviving plants.

Selection of putative tetraploid plants using stomatal length

The mean stomatal length of control plants (diploid) varied significantly among different cultivars (Table 9). The stomatal size of cultivars BM, RQ and G were significantly ($p=0.05$) larger than those of PST and RG. The stomatal size of tetraploids were compared with that of the diploids of the same cultivar.

For each hybrid cultivar, the mean stomatal length of colchicine-treated plants was slightly larger than that of control plants. The minimum stomatal length from colchicine-treated and control populations was similar but the maximum stomatal length and SD were much larger in colchicine-treated population than in control population (Table 9). A comparison between control and colchicine-treated populations for stomatal length showed overlap in plants with smaller-sized stomata. Colchicine-treated populations, however, have a number of plants with larger stomata (Fig. 4). Of 565 colchicine-treated plants of nine cultivars, 110 plants were selected as putative tetraploid plants (Table 10) based on stomatal size (selection method in

Section 2.8). This was consistent with the hypothesis that plants recovered from colchicine-treated cultures were a mixture of diploids and polyploids. The stomata of these plants were clearly larger than those of the control plants (Fig. 6. A, 6.B).

Confirmation of tetraploids by chromosome counting

There are 32 chromosomes in all diploid *Zantedeschia* species (Earl 1957, Chapter 4). Chromosome numbers were counted in 45 of those 110 putative tetraploid plants. Of these plants only five were diploids, 38 were tetraploids. The remaining two plants were mixoploids; they were predominantly tetraploid with a few octoploid cells (Table 10). Fig. 6 shows 32 chromosomes in a root-tip cell of diploid control plant and 64 chromosomes in a root-tip cell of colchicine induced tetraploid plant. Thus 91% (40 out of 44) of the putative tetraploids were shown to be tetraploids or tetraploids/octoploids. Stomatal size was thus a good indicator for ploidy level in *Zantedeschia*.

Stomatal size in mature diploid and tetraploid plants of *Z. 'Black Magic'*

From mature 'Black Magic' plants, data was collected and analysed as described in Section 2.9. Stomatal size was significantly different between ploidy levels ($p=0.001$), between plants within a ploidy ($p=0.05$), between leaves within a shoot ($p=0.01$), and between samples within a leaf ($p=0.01$) (Table 11). Ploidy level, however, was the major variance factor and contributed 77.8% of the total variance (Table 11). Other factors, plants, leaf and sample, had only a minor effect on the variation of stomatal size. The stomatal size of each tetraploid sample was more than 2X SD greater than the control mean with only one exception. The stomatal size of each diploid sample and that of each tetraploid sample were well separated, except for a few samples which overlapped at 51 μm (Fig. 5.A). A pooling of four samples from a single shoot showed that the stomatal size of each tetraploid shoot was more than 2X SD greater than the control mean. The stomatal size of each diploid shoot and that of each tetraploid shoot were then completely separated (Fig. 5.B). These results demonstrated that most tetraploid plants can be distinguished from diploid plants using only one sample from each plant. Where tetraploid plants could not be clearly resolved by individual samples, the pooling of four samples from each shoot clearly distinguished these tetraploid plants from the diploid plants. The mean stomatal size of the young diploid 'Black Magic' plants (45.8 μm , Table 9) was the same as that of mature diploid plants (45.4 μm).

Table 9. Stomata size (μm) of control and colchicine-treated plants of nine cultivars

Cultivar	Control					Colchicine				
	N*	Mean**	SD	Min.	Max.	N	Mean	SD	Min.	Max
PST	9	35.3a	4.1	29.1	41.8	31	36.8	5.1	27.8	49.3
RG	9	36.8a	2.5	33.1	40.4	34	42.5	8.6	29.1	66.8
PPK	8	37.9ab	5.0	32.0	46.4	37	41.4	9.1	28.1	61.6
RE	5	40.1abc	5.9	32.3	48.4	46	45.5	7.0	33.1	61.8
RB	7	41.5abc	4.0	35.1	46.1	43	44.3	11.1	30.7	74.6
GS	8	41.9abc	5.5	36.3	50.3	78	43.9	8.5	31.4	70.4
G	10	43.6bc	4.1	37.5	49.5	67	51.9	10.8	33.0	77.6
RQ	15	44.8c	5.5	35.9	52.1	167	47.5	7.1	34.5	68.8
BM	8	45.8c	3.8	39.5	50.8	62	47.1	9.4	35.1	79.0

* Number of plants tested.

** Means followed by the letter are not significantly different ($p=0.05$) when analysed by ANOVA.

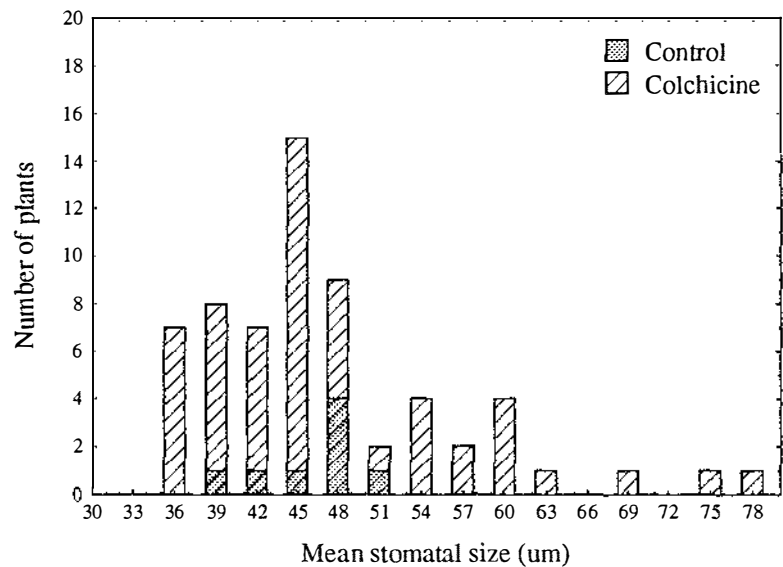


Fig. 4. Distribution of mean stomatal size of 8 control and 62 colchicine-treated plants of Z. 'Black Magic'

At eight weeks after the plants were transferred to a greenhouse, ten stomata were measured on one leaf of each plant. The mean length of the ten stomata was used in this graph.

Table 10. Tetraploid plants produced from nine cultivars

Cultivar	Time on colchicine medium	Plants checked for stomatal size	Plants with >2XSD stomata	Tetraploid plants confirmed by chromosome counts *
PST	2 days	31	4 (12.9%)	1/1
RG	2 days	34	5 (14.7%)	0/1
PPK	1 day	37	8 (21.6%)	4/4 **
RE	4 days	46	7 (15.2%)	3/3
RB	1 day	43	7 (16.3%)	3/3
GS	4 days	78	13 (16.7%)	11/11
G	2 days	67	28 (41.8%)	5/6 **
RQ	4 days	167	24 (14.4%)	5/7
BM	2 days	62	14 (22.6%)	8/8
Total		565	110 (19.5%)	40/44

*: Number of tetraploids/number of plants checked, **: One plant had a few octoploid cells.

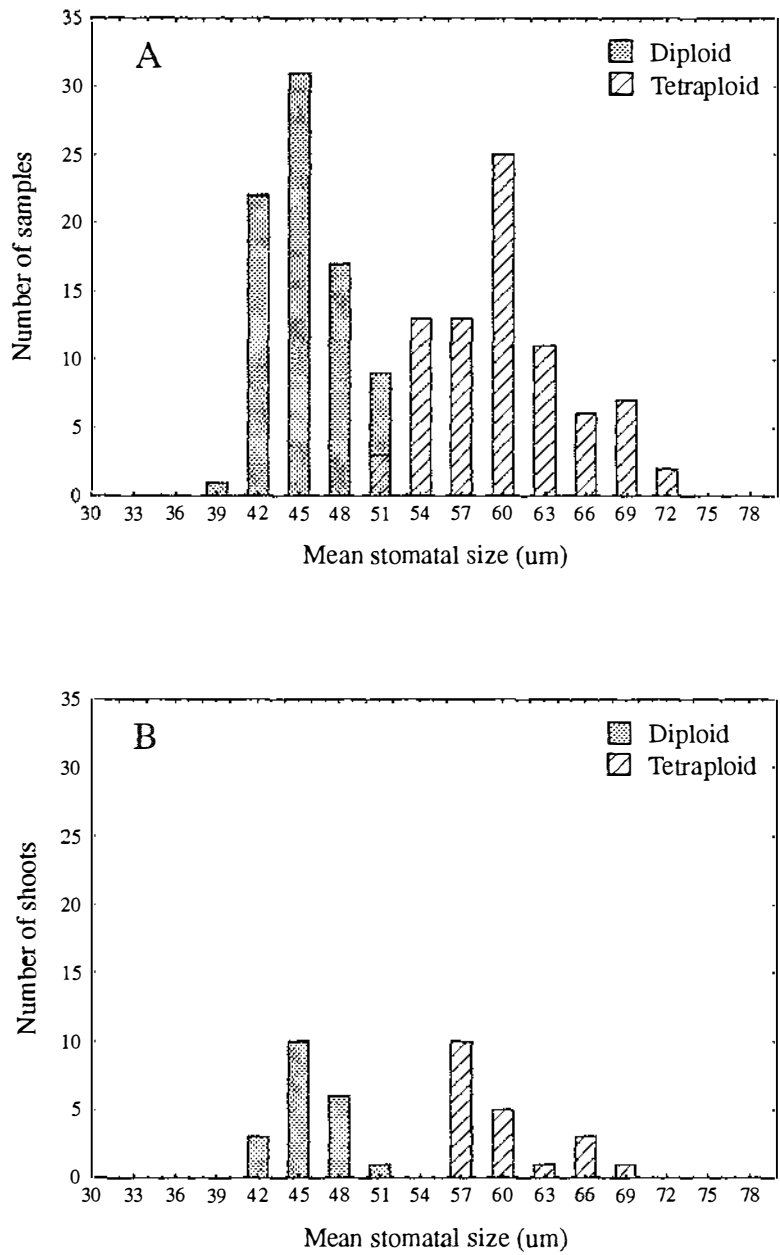


Fig. 5. Stomatal size distribution of diploid and tetraploid plants of *Z. 'Black Magic'*

(A): distribution of stomatal size of 80 diploid and 80 tetraploid samples.

(B): distribution of stomatal size of 20 diploid and 20 tetraploid shoots.

Samples were taken at flowering time. Five shoots were chosen from each of four diploid and four tetraploid plants. Two leaves were selected from each shoot and two samples were taken from each leaf. Twenty stomata were measured from each sample.

Table 11. Variance analysis of stomatal size of diploid and tetraploid 'Black Magic'

variance sources	degree of freedom	sum of square	mean square	F value	variance component	% of total variance
Ploidy (2x/4x)	1	110731.9	110731.9	126.19 ^{***}	68.6	77.9
Plant (within ploidy)	6	5264.9	877.5	2.46 [*]	1.3	1.5
Shoot (within plant)	32	11391.8	356.0	1.24 ^{ns}	0.9	1.0
Leaf (within shoot)	40	11432.2	285.8	4.77 ^{**}	5.7	6.4
Sample (within leaf)	80	4759.7	59.5	6.52 ^{**}	2.5	2.9
Error	3040	27735.0	9.1		9.1	10.4

*** p=0.001, **: p=0.01, *: p=0.05, ns: not significant.

Some other characters of tetraploid plants

Measurements of leaf thickness and leaf weight (Section 2.10) were made for tetraploid plants and diploid control plants. The mean and standard error (SE) of leaf thickness and weight of diploids and tetraploids from five cultivars are listed in Table 12. By T test, the mean of leaf thickness of the tetraploids was significantly different ($p \leq 0.05$) from that of the diploids in cultivars GS, RE, PPK and BM, but not in RQ. The mean of specific leaf weight of the tetraploids was significantly different ($p \leq 0.05$) from that of the diploids in cultivars GS, BM and PPK, but not in RE or RQ (Table 12). The leaf thickness and weight of approximately 50% of the tetraploid plants were more than 2X SD greater than the control (diploid) mean. In summary, leaf thickness and specific leaf weight were generally greater in the tetraploid plants although these features were not always reliable for distinguishing tetraploid plants from diploid plants.

Pollen from tetraploid plants was larger in size than that from diploid plants of the same cultivar although the pollen morphology was the same in tetraploid and diploid

plants (Fig. 6.E, 6.F). Pollen of some tetraploid plants was used for pollination and early development of embryos and endosperm was seen (Section 3.5). This result indicated that pollen of tetraploid plants was viable. In ‘Black Magic’, the flower stem of tetraploid plants was apparently thicker than those of diploid plants (Fig. 7). In some cultivars, such as ‘Golden Sun’, the spathe of tetraploid plants was thicker than those of diploid plants.

Table 12. Leaf thickness and specific leaf weight of diploid and tetraploid plants of five cultivars ^a

cultivar	diploid			tetraploid		
	N	LT	SLW	N.	LT	SLW
		M±SE	M±SE		M±SE ^b	M±SE ^b
GS	6	487 ± 16.4	37.5 ± 1.74	10	600 ± 18.7 [*]	45.9 ± 1.24 [*]
BM	6	417 ± 24.9	32.5 ± 2.13	6	508 ± 10.6 [*]	38.1 ± 0.59 [*]
RE	6	509 ± 16.4	42.0 ± 1.84	4	588 ± 10.7 [*]	46.5 ± 1.13 ^{ns}
RQ	6	556 ± 27.2	40.9 ± 2.68	4	622 ± 22.4 ^{ns}	48.5 ± 2.02 ^{ns}
PPK	4	404 ± 19.5	32.6 ± 1.28	2	564 ± 6.3 [*]	45.5 ± 2.10 [*]

a: Method was described in Section 2.10, tetraploids were all conformed by chromosome counts.

N: Number of plants tested

LT: Leaf thickness (μm)

SLW: Specific leaf weight (mg/cm²)

b: The means of tetraploids were compared with the means of diploids of the same cultivar using the two sample T test of Minitab (based on the pooled standard deviation). ^{*}: significantly different (p ≤ 0.05), ^{ns}: not significantly different (p ≥ 0.05).

Fig. 6. Stomata, chromosomes and pollen of diploid and tetraploid Z. 'Black Magic'

(A, C, E): diploid

(B, D, F): tetraploid

Bar: 40 μm in (A) and (B)

Bar: 5 μm in (C) and (D)

Bar: 25 μm in (E) and (F)



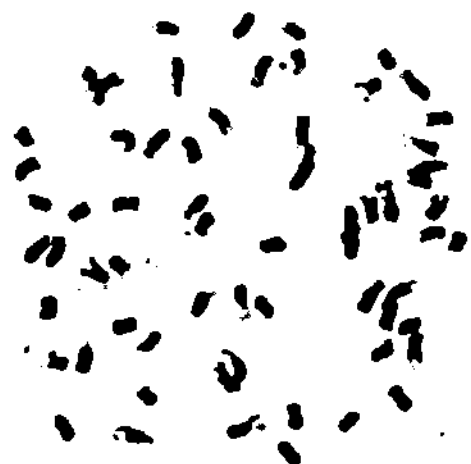
A



B



C



D



E



F



Fig. 7. Diploid plants and tetraploid plants of Z. 'Black Magic'
Show the thicker flower stems in tetraploid plants.

3.2. Colchicine treatment of multiplying shoots of summer-flowering species

In order to produce cultures suitable for tetraploid induction, buds were dissected from sporting tubers of *Z. pentlandii* (PE), *Z. elliottiana* (EL) and *Z. rehmannii* (RE). Dissected buds greatly enlarged at four weeks after the initial culture on a medium with 0.3 mg/l BA, but there was no multiplication (Fig. 8.B). Multiplication, however, occurred for PE and EL after these buds were split longitudinally and transferred to a medium with 3 mg/l BA (Fig. 8.C). Two subcultures of PE and EL were made on a medium with 3 mg/l BA to obtain enough shoots before colchicine treatment. After one day treatment on a medium with 0.05% colchicine and transfer to a colchicine-free medium with 3 mg/l BA, many shoots died within the first subculture. The survival rate on different plates varied from 30% to 70 %. On the same medium, a second subculture was performed to multiply the surviving shoots (Fig. 9). After shoot elongation and rooting (Section 2.4), plantlets of PE and EL were transplanted in a greenhouse. RE did not multiply in culture and therefore was not treated with colchicine.

Stomatal length measurements (Section 2.7) were made from control (diploid) and colchicine-treated plants of PE and EL. The results are summarised in Table 13. By comparing stomatal size (Section 2.8), 29 and three putative tetraploid plants were selected from PE and EL respectively (Table 13). Chromosome counts were not made for these plants.

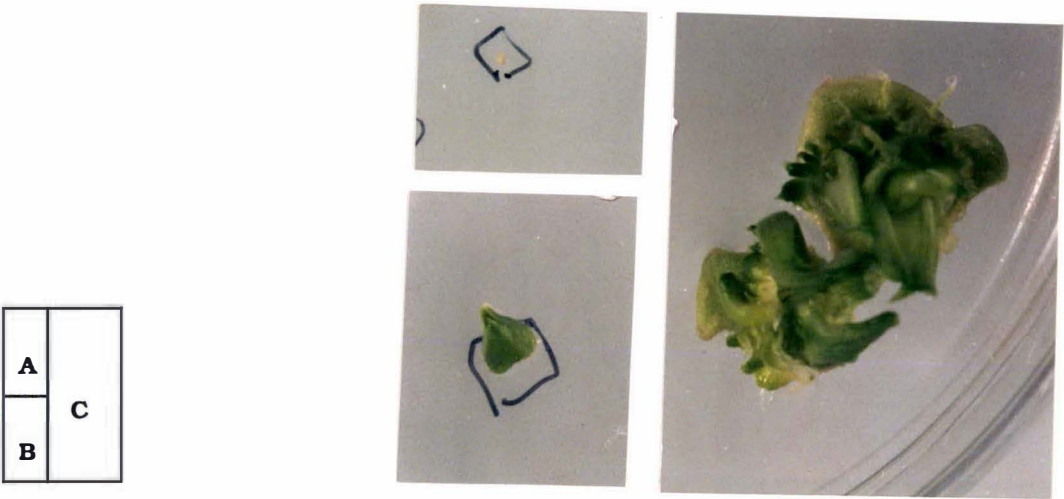


Fig. 8. Multiplication from bud of *Z. pentlandii* in vitro
(A): a dissected bud at the beginning of culture.
(B): a greatly enlarged bud after four weeks on medium with 0.3 mg/l BA.
(C): multiple shoots induced from longitudinally split buds on (B) after two weeks on medium with 3 mg/l BA.

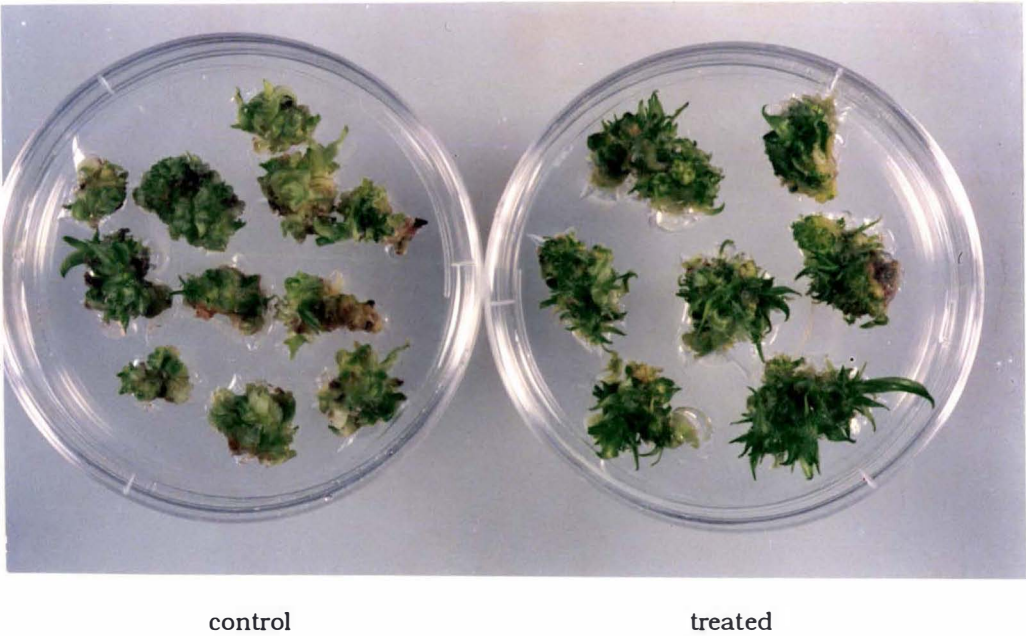


Fig. 9. Control and colchicine-treated cultures of *Z. pentlandii*
The photograph was taken at the end of the second subculture, two months after the treatment with 0.05% colchicine for 24 hours.

Table 13. Stomatal size (µm) of control and colchicine-treated plants and putative tetraploids of four *Zantedeschia* taxa

Plant	Control					Colchicine						
	N*	Mean	SD	Min.	Max.	N	Mean	SD	Min.	Max	Putative 4X**	
PE	12	33.3	2.4	29.6	36.9	120	36.1	6.8	26.3	58.3	29	(24.2%)
EL	10	31.9	2.8	28.4	35.5	8	37.5	6.0	32.1	50.0	3	(37.5%)
CHR	6	35.1	1.6	33.3	37.5	55	39.6	6.4	27.6	55.0	22	(49.0%)
CHI	7	39.0	2.4	35.8	41.6	102	45.5	7.6	33.1	66.0	54	(52.9%)

*: Number of plants tested.
 **: Number of putative tetraploid plants based the stomatal size as described in Section 2.8.

3.3. Colchicine treatment of embryos *in vitro*

Mature embryos dissected from dry seeds of *Z. aethiopica* 'Childsiana' were about 2.2 mm in length. The embryos started to increase in size immediately after they were cultured on a medium with no BA or 0.3 mg/l BA. Embryos reached about 3 mm after four days in culture. After a week, cotyledons were elongated and had turned green. Another week later, the first leaf emerged and roots formed. When embryos were treated with colchicine at four days in culture (Section 2.5), the cotyledon elongation was inhibited. The formation of root and the first leaf was totally inhibited or delayed with restricted growth (Fig. 10). Two-day treatment inhibited growth more strongly than one-day treatment. Although limited root and leaf development occurred in some colchicine-treated embryos, these embryos eventually died in subcultures.

The embryos of summer-flowering, hybrid-cultivar 'Chromatella' were slightly larger than those of 'Childsiana', about 3.0 mm long. The germination and response to the colchicine treatment *in vitro* of 'Chromatella' embryos were similar to those of 'Childsiana' embryos (Fig. 10), but a few surviving 'Chromatella' embryos generated multiple shoots on subculture. These shoots were elongated, rooted and transplanted in a greenhouse. Stomatal data were obtained (Table 13) by the method used previously (Section 2.7, 2.8). Of 55 plants generated from the multiple shoots, 22 (49%) were selected as putative tetraploids (Table 13).

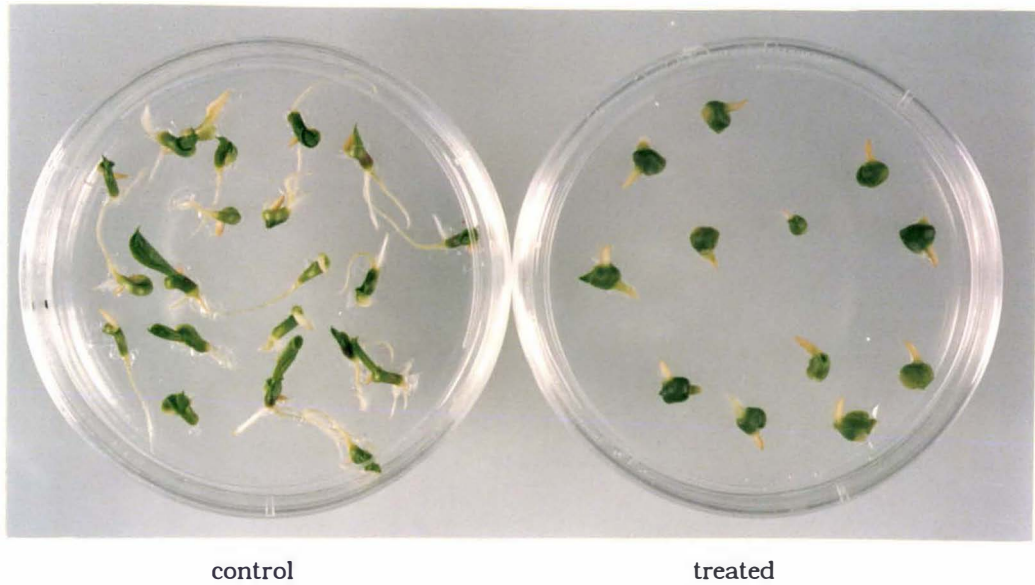


Fig. 10. Colchicine treatment of embryos *in vitro*

Embryos of 'Chromatella' were treated for two days on a medium with 0.05% colchicine. Photograph was taken at 15 days after colchicine treatment. 'Childsiana' embryos showed similar results as in this photograph.



Fig. 11. Germinating seeds of *Z. 'Chromatella'*

Seeds were photographed after five weeks in germination condition and were at different germination stages. 'Childsiana' seeds had a similar germination.

3.4. Production of tetraploid plants by colchicine treatment of germinating seeds

During the course of seed germination of ‘Childsiana’ (CHI) and ‘Chromatella’ (CHR), the cotyledon elongates and pushes a part of itself with the plumule and radicle out of the endosperm and seed coat at the micropyle end. The haustorial tip of the cotyledon remains in the endosperm. The cotyledon continues to enlarge, turns green, then finally opens. After the cotyledon is open, the first leaf and root emerge (Fig. 11). Colchicine treatment (Section 2.6) was applied after the cotyledon emerged and before it opened (Fig. 11).

Table 14. Plants of CHI generated from colchicine treatment of germinating seeds showing variation in stomatal size among leaves ^a

Plant No	chromosome number	Stomatal size (µm) of individual leaf	
		<control mean + 2xSD ^b	>control mean + 2xSD
CHI T3	4x	39.9	45.5 53.3
CHI T37	- ^c	33.3 37.2	55.6
CHI T38	4x	33.3 37.1	45.5
CHI T41	4x		49.3 49.8 50.9 51.1
CHI T53	-	33.8 36.3 40.6	49.9
CHI T58	4x	34.4 37.8 37.9	
CHI T65	-	37.0 41.0	54.6
CHI T84	-	36.3	44.6 54.6
CHI T88	-		45.3 46.4 47.0 51.9 55.6

a: The nine plants were generated from colchicine-treated seeds and had stomata more than 2X SD greater than the control mean at the first screening for tetraploids.
b: The control mean which was obtained from eight leaves of four control plants was 39.9 µm with a SD of 2.2 µm.
c: chromosomes not observed.

From CHI, 155 germinating seeds were treated with 0.05% colchicine for one day and 116 plants were established in a greenhouse. Stomatal size measurements (Section 2.7) were made from these 116 plants and 54 (52.9%) putative tetraploid plants were selected (Table 13). Chromosome counting (Section 2.11) was made from seven putative tetraploid plants and six plants were confirmed as tetraploids. After the plants flowered, 3 to 5 leaves from each of nine plants were sampled for stomatal

measurements. Two plants had all leaves with stomata more than 2X SD greater than the control mean. Six plants had some leaves with stomata more than 2X SD greater than the control mean and other leaves with stomata less than 2X SD greater than the control mean. One plant had stomata less than 2X SD greater than the control mean in all three leaves investigated (Table 14). This plant had earlier been shown to have a tetraploid chromosome number. Thus the plant must have been a chimera.

One leaf of each of four plants of CHI was measured for stomatal length from leaf opening to four days after the leaf fully opened. There was no tendency for increasing stomatal size as a leaf matured (data not shown).

From CHR, 30 germinating seeds were treated with 0.05% colchicine for one day and 14 plants were established in a greenhouse. One plant had stomata more than 2X SD greater than the control mean and had 64 chromosomes.

3.5. Triploid plants produced from 2X/4X crosses with the aid of embryo rescue

Pollination and development of ovaries and ovules

Eight inflorescences of diploid plants were pollinated with pollen from tetraploid parents (Table 15). Diploid *Z. elliottiana* were self-pollinated at the same time for a control. Development of embryos and endosperms was examined at 8 weeks after pollination (WAP). Ovaries from 2x/4x crosses were found smaller than ovaries from 2x/2x crosses (Fig. 12). Ovaries of 2x/4x crosses had fewer seeds than the controls. Seeds of 2x/4x crosses were similar in size to the controls and most had a well developed seed coat (outer-integument). The inner-integument was thin, yellow or brown. However the endosperm was poorly developed or had degenerated (watery). It was difficult to see an embryo in these poorly developed endosperms under a dissection microscope. Three seeds were dissected with development close to that in the controls. The development of these seeds was assumed as the result of self-pollination. In these three seeds, as well as in the seeds of the controls, the endosperm was larger and semi-soft, and embryos were elongated, about 3 mm in length.

Embryo culture

Cultures are listed in Table 15. Although cultures increased in size, most of them did not generate any plants except for one embryo from inflorescence No. 6, and one callus-like tissue from inflorescence No. 7. From inflorescence No. 6, three embryos

were cultured. Two months later, one of the embryos generated a number of buds through a callus phase. These buds multiplied, elongated and rooted in culture. A number of plants were established in a greenhouse. Another embryo generated a few buds on callus after several subcultures but the buds did not grow to plants. The third embryo generated white, soft callus but did not generate any buds. From inflorescence No.7, none of the 16 cultured embryos generated any plants. In one case, a callus dissected from the embryo sac produced multiple buds in culture. At the time of dissection, the embryo sac was watery and contained a white, hard callus. The watery part may have formed from degeneration of the endosperm. The callus was probably of embryo origin. No regeneration was ever observed from endosperm tissue.

Chromosome number

Chromosome counts were made for two plants regenerated from an embryo of inflorescence No. 6 and three plants regenerated from a callus of inflorescence No.7. A number of mitoses were observed. They were all triploid. Fig. 13 clearly shows 48 chromosomes in a root-tip cell of a triploid plant. Other plants regenerated from these two inflorescences could be triploids as they regenerated from the same embryo or callus.

Table 15. Number of seeds formed and embryos cultured from each inflorescence of 2x/4x crosses

No.	Crosses	No. of ovaries with seeds	No. of seeds	Material on culture
1	D X GS41	33	40	9 embryos
2	WP X GS41	none	none	none
3	C X GS41	11	19	11 embryos
4	D X RE40	none	none	none
5	D X RE40	50	80	none
6	D X RE40	20	48	3 embryos
7	EL X GS53	36	50	16 embryos, 5 calli
8	EL X GS53	none	none	none



Fig. 12. Ovaries of *Z. elliotiana* (2x) pollinated with pollen of 'Golden Sun' (4x) (left) and self-pollinated (right)

The photograph was taken at eight WAP.



Fig. 13. Forty-eight chromosomes in a root-tip cell of a triploid plant

The plant regenerated from inflorescence No. 6 of 'DX' (2x) X 'Red Emperor' (4x).

4. Discussion

Tetraploid production

By incorporating colchicine treatment into the micropropagation system (Cohen 1981) based on multiplying shoots *in vitro*, tetraploid plants were produced from 11 summer-flowering *Zantedeschia* cultivars and species. Although only 1 or 2 petri-dishes containing multiple shoots of each cultivar or species were initially treated with colchicine, up to 28 putative tetraploids were selected from a single cultivar (Table 10). Chromosome counts confirmed the tetraploid status of 90% of these plants that were checked. In Table 10, the two mixoploid plants which had predominantly tetraploid cells and a few octoploid cells may be tetraploid/octoploid chimeras caused by colchicine treatment or tetraploid plants with a few endo-octoploid cells. Endo-tetraploid cells were also found in diploid plants (Chapter 4). There were five putative tetraploid plants which had large stomata but had a diploid chromosome number (Table 10). They may be completely diploids and were misdetermined as tetraploids by stomatal size observation. Some misidentification would be expected because of the variation in stomatal size within ploidy level. These five may also be periclinal chimeras with tetraploid leaves and diploid roots, or mericlinal (sectoral) chimeras in which the tetraploid cells in roots were not observed. Chromosome counting is unequivocal for ploidy of the cells directly observed but only inferred with respect to the rest of the plant. Four mature tetraploid plants of 'Black Magic' were intensively investigated by stomatal measurements. Of 80 samples tested, 79 displayed a tetraploid stomatal size by comparing the stomatal size of individual samples with the diploid control mean (Section 3.1). Therefore there was little sign of chimerism in these plants.

Colchicine treatment of multiple shoots *in vitro* was not suitable for *Z. rehmannii* and *Z. aethiopica* because they were difficult to multiply on the media. *In vitro* treatment of embryos was not successful because colchicine inhibited plant growth and caused death on the medium. A reduction of colchicine concentration in the medium and/or treating period may overcome this problem.

By colchicine treatment of germinating seeds, a number of tetraploid plants were produced from *Z. aethiopica* 'Childsiana' and summer-flowering hybrid 'Chromatella' although some of them may be chimeras. Of nine putative tetraploid plants of 'Childsiana', six showed some leaves with diploid stomatal size and other leaves with

tetraploid stomatal size (Table 15). It seemed that colchicine treatment of germinating seeds produced many chimeras as has been reported by many authors (Arisumi 1964, Pryor & Frazier 1968, Semeniuk & Arisumi 1968, Ackerman & Dermen 1972).

Colchicine treatment of multiple shoots *in vitro* gave a better efficiency of tetraploid production and a lower frequency of chimera formation than colchicine treatment of germinating seeds. Several possible reasons for this are that a large number of multiple shoots were treated with colchicine in one or two petri-dishes, surviving shoots after colchicine treatment multiplied rapidly and chimeras would tend to separate during the subsequent multiplication stage.

Colchicine treatment of multiple shoots *in vitro* are likely to produce tetraploids with the same genetic identity as the parent plant. Colchicine treatment of callus and suspension cells are more likely to induce genetic variation in addition to the chromosome doubling. Also, one is not confronted with genetic segregation by this method, unlike the treatment of seeds which are the product of sexual reproduction. In addition, the multiplication system could produce a somaclone of a tetraploid plant derived from a certain cultivar or hybrids. A similar approach could be used to produce tetraploid plants in other genera if a micropropagation system can be developed.

Tetraploid selection

The strategy of tetraploid selection in this study was to screen a population of colchicine-treated plants with stomatal measurements for putative tetraploids and confirm tetraploidy with chromosome counting. The method used for stomatal measurements in this study was simpler than those used in previous studies (Speckmann *et al.* 1965, Tan & Dunn 1973, Borrino & Powell 1988, Przywara *et al.* 1988) because the chlorophyll extraction step was omitted and the impression of epidermis was removed by sellotape instead of using forceps to remove the epidermis. The stomatal image, however, was clear (Fig. 7.A, 7.B) and the samples can be stored at room temperature indefinitely.

Positive and negative features for stomatal size as a ploidy level indicator were found in *Zantedeschia*. Positively, stomatal size measurement was simple and allowed rapid screening of a population for tetraploids. Stomatal sizes did not vary with leaf developmental age (Section 3.4) nor with the age of plant (Section 3.1, for 'Black

Magic'). Even using a sample of 10 stomata to select putative tetraploids, 89% of the plants selected as putative tetraploids were later confirmed to be tetraploids by chromosome counts (Table 10). On the negative side, ploidy of some plants could be misdetermined by stomatal measurements because of the overlap of stomatal size between some samples of diploid and tetraploid plants. Although an increase in number of stomatal sample for each plant improves the accuracy of tetraploid selection, this may not be suitable for small young plants. The reliability of stomatal size as a ploidy indicator varies among plant species, and is poor in some species, as in *Trifolium riograndense* (Schifino & Fernandes 1987). In *Zantedeschia*, the stomatal size of diploid samples overlapped with tetraploid samples (Fig 2A). Similar overlap has also been observed in kiwifruit (Przywara *et al.* 1988) and barley (Borrino & Powell 1987). This type of overlap may be caused by stomatal size variation within ploidy level (Table 11, Schifino & Fernandes 1987). This variation, in *Zantedeschia*, was statistically significant but much smaller than the variation between ploidy levels (Table 11). This feature made ploidy level determination by stomatal measurement practicable but not infallible. Therefore the procedure, screening for tetraploids by stomatal measurements and confirming tetraploidy by chromosome counting, which was used hereby seemed an efficient and accurate way for tetraploid identification.

Triploid production

Crosses between species within section II of the genus *Zantedeschia* are usually compatible at the diploid/diploid level (New & Paris 1967, Chi 1990). Strong postfertilisation incompatibility was shown between diploid and tetraploid within section II in this study (Section 3.5). This incompatibility may be caused by changing the gene balance as described by the endosperm balance number (EBN) hypothesis (see Section 2.3 of Chapter 3). From an anatomical point of view, the origin of triploid plants rescued from inflorescence No. 7 was not clear because the initial cultured tissue was a callus formed within the embryo sac. However, an embryonic rather than an endospermic origin was assumed for two reasons. Firstly, the endosperm tissue of $2x \times 4x$ was unlikely to be triploid. Secondly, no plants had been regenerated from any endosperm on this type of medium even though over one thousand endosperms had been cultured (refer to Chapter 6 and Chapter 7).

CHAPTER 6. Interspecific hybridisation between the two sections Of *Zantedeschia*

1. Introduction

As mentioned in Chapter 2, there are some complementary characters between the two sections of *Zantedeschia* which are interesting to plant breeders. Sexual hybridisation is the most commonly used approach to combine different characters from different species. Other approaches, such as protoplast fusion and genetic transformation, could be also used to combine different genetic characters. Genetic transformation is a long term approach because it requires gene identification, cloning, and transformation. Protoplast fusion has the advantage of overcoming prefertilisation incompatibility but postfertilisation incompatibility may still prevent recovery of hybrids (Bravo & Evans 1985). Individual species may require different protoplast regeneration conditions which are normally difficult to determine. Sexual hybridisation is a relatively simple and straightforward approach. First, it is appropriate to attempt sexual hybridisation.

Traub (1949) reported that attempts to produce hybrids between the two sections of *Zantedeschia* failed with no viable seed formation. A recent study has found that the barriers for the seed formation involve postfertilisation incompatibilities (Chi 1990). Chi crossed the cultivars 'Best Gold' and 'Chromatella' (in section II) with a paternal parent *Z. aethiopica* 'Childsiana' (in section I). Most embryos did not develop beyond the globular stage and endosperm development was poor. Few embryos reached 1 mm in length. From three of these elongated embryos, albino shoots were produced *in vitro* (Chi 1990). In the present study, the ultra-structure of plastids of some albino plants was compared with the ultra-structure of plastids from green shoots *in vitro* in an attempt to identify the block to chloroplast development. Letty (1973) noted that the flowering seasons of plants from different sections of *Zantedeschia* is different. Furthermore, pollen of *Z. aethiopica* has a very short storage life. Pollen viability in a sealed tube stored at 4 °C was reduced to 10% within two weeks (Chi 1990). The present study grew plants in greenhouses to manipulate flowering time and tested the effects of further genotypes, cross direction and ploidy level on the crossability between the two sections of *Zantedeschia*.

2. Material and methods

2.1. Plant material

From *Z. aethiopica* (section I), three genotypes were used in the crosses. A strain typical of *Z. aethiopica* growing in New Zealand (AE) was collected from Massey University around the pool beside the Veterinary Tower. A strain of *Z. aethiopica* with spotted leaves (AE_{sp}) was collected from a gully near Palmerston North. The third genotype was *Z. aethiopica* 'Childsiana' (CHI), a dwarf cultivar described by Traub (1949) and Bailey & Bailey (1976). From section II, *Z. elliotiana* (EL) and the following hybrid cultivars were used in crosses. These cultivars were: 'Chromatella' (CHR) (CHR is closely related to *Z. albomaculata*), 'Black Magic' (BM), 'Golden Sun' (GS), 'Galaxy' (G), 'Pacific Pink' (PPK), 'Purple Star' (PST), 'Red Beauty' (RB), 'Red Emperor' (RE), 'Red Gold' (RG), 'Rose Queen' (RQ), 'Pink Satin' (PS), 'Pink Persuasion' (PP), 'Best Gold' (BG) and 'Majestic Red' (MR). Induced tetraploid plants (Chapter 5) from CHI, GS, BM, RE, PPK, G, PP were also used in crosses. It was later found that the tetraploid plants of CHI were chimeric, producing both tetraploid and diploid shoots on the same plants.

Plants were grown from seed of AE and AE_{sp}, from rhizomes of CHI and tubers of genotypes of section II. The tubers were harvested at the end of each growing season and stored at 16 °C until the next planting. At the time of replanting, tubers were dipped for approximate 30 seconds in a solution of 50 mg/l gibberellic acid (GA₃ or GA₄ + 7) to increase flower production. All plants were grown in pots in greenhouses. The potting mix used is described in the Appendix 2.

2.2. Pollen germination

In vitro pollen germination tests used a medium containing 100 g/l sucrose, 20 mg/l boric acid, 100 mg/l CaCl₂, and 7 g/l agar at pH 6 (Chi 1990) was used. The medium was autoclaved at 1.1 kg/cm² for 15 minutes. Approximately one millilitre of the medium was dropped on to a microscope slide and allowed to solidify. Freshly released pollen was collected from inflorescences of AE, AE_{sp}, GS and GS_{4x} and spread onto the medium on individual slides. The slides were placed on wet filter paper in covered petri-dishes and incubated at 20 °C. Three fields of view (80-300 grains/field) were examined from each slide to determine pollen germination after overnight incubation.

2.3. Pollination

All pollinations were made using fresh pollen. Pollen was applied to stigmas with a small paint brush. The brush was washed in 70% acetone to kill pollen between pollinations (Chi 1990). If plants of summer-flowering species or hybrids were used as female parents, emasculation was achieved by removing the male zone and staminodes in the upper portion of the female zone. If *Z. aethiopica* was used as the female parent, it was not necessary to emasculate provided pollination was made on recently opened inflorescences (New 1964). After pollination, inflorescences were covered with paper bags tied with a fine wire to protect the inflorescences and to prevent accidental pollination. For an individual inflorescence, pollinations were repeated two or three times at two-day intervals.

Crosses were made in three different periods. The first pollination season was from 13th April to 18th June 1990. The second pollination season was from 5th September to 18th November 1990 and the third season was from 12th February to 22th May 1991. All crosses are listed in Appendix 3 and a summary of seed formation from the crosses is given in Table 16.

2.4. Embryo rescue

Ovaries were collected at 4, 5, 6, 7, or 10 weeks after pollination (WAP). Ovules were removed after the ovaries were sterilised with sodium hypochlorite as described in Chapter 5. Both outer and inner integuments were dissected away and the small embryo embedded in the endosperm was cultured on MS medium (see the method section of Chapter 5) supplemented with 0.3 - 3.0 mg/l BA (6-benzyladenine). In some experiments the effects of 0.3 mg/l NAA (1-naphthalene acetic acid) and 400 mg/l CH (casein hydrolysate) were tested as described in the Results Section. In some cases, isolated embryos were cultured as indicated in the Results Section.

2.5. Observation of plastid ultra-structure

In vitro grown shoots of albino hybrids of AE X EL (Table 7), CHR X CHI No.47 (Chi 1990) and a normal green genotype WP51 (a cultivar in section II) were cut close to the base and subcultured on a medium with 1.0 mg/l BA in the dark or light for five weeks. Leaf tissue from these cultures, as well as from plants of CHI and CHR growing in a greenhouse was collected. The leaf tissue was cut into squares of about 0.2 X 0.2 cm and fixed in 3% glutaraldehyde, 2% formaldehyde in 0.1 M phosphate buffer, pH 7.2. Leaf sections continued to float even when the vials were evacuated with a water aspirator. Addition of Triton-X100 to a final concentration of 0.1% to the

fixative reduced surface tension and allowed the sections to sink following a small quantity of evacuation with the water aspirator. The fixative was then replaced with a fresh solution without Triton and left overnight at 4 °C. The samples were post-fixed in 1% OsO₄ in 0.1 M phosphate buffer, pH 7.2, followed by dehydration in a graded acetone series, infiltration and embedding in Polarbed 812 epoxy resin. Thin sections were cut on a diamond knife using a Reichert Ultracut E ultramicrotome and stained with uranyl acetate and then lead nitrate. Stained sections were observed and photographed in a Philips 201c Transmission Electron Microscope (TEM).

3. Results

3.1. Flowering, fertilisation and development of ovary and ovule

Simultaneous flowering of plants from the two sections of *Zantedeschia* was achieved by growing plants in a greenhouse. Seedlings of *Z. aethiopica* (AE) first flowered when they were about ten months old and continued to flower all year round. The flowering time of *Z. aethiopica* 'Childsiana' was extended by dividing the rhizomes and growing the plants in a greenhouse. The flowering time of plants in section II was controlled both by planting time and the use of GA treatment. Plant normally flowered about ten weeks after planting.

Stigmas which appeared wet before pollination shrank and became dry within a few days of pollination. Ovaries expanded rapidly over a period of about three weeks. In the compatible crosses, ovules formed mature seeds after ten weeks. In the incompatible crosses, no mature seeds formed, but ovules developed slowly into abnormal seeds. Most of these seeds had a soft and watery endosperm. Some seeds were empty within the thick seed coat through the complete degeneration of the endosperm. The ovaries were smaller in incompatible crosses than in compatible crosses because of poor seed development and fewer seeds. The ovaries of incompatible crosses were very similar to those of 2x/4x crosses in Fig. 12. If fertilisation did not occur, ovaries expanded for approximately two or three weeks but expansion was always less than for fertilised ovaries. At four WAP, inflorescences containing no fertilised ovaries began to shrivel and later die. Fertilised ovaries could be easily distinguished from unfertilised ovaries by four WAP.

Table 16. Effect of pollination seasons on seed formation

crosses	pollination season	total inflorescences pollinated	proportion of inflorescences with seed development (%)	average number of seeds per inflorescence with seed development
I X II	first	30	23.4	16
	second	138	65.2	41
	third	18	27.8	10
II X I	first	37	21.6	9
	second	24	95.8	69
	third	100	6.0	23
I X I	first	10	40.0	81
	second	39	94.9	98
	third	5	40.0	66
II X II	first	23	52.2	18
	second	4	100.0	100
	third	49	30.6	16
all crosses	first	100	31.0	23
	second	205	75.6	60
	third	172	16.3	17

I: section I, *Z. aethiopica*. II: section II, summer-flowering *Zantedeschia* species and hybrids.

Much better fertilisation was achieved in the second pollination season than in the first and third pollination seasons based on the proportion of inflorescences with at least one seed and the average number of seeds per inflorescence (Table 16). Overall, in the second pollination season, 75.6% of pollinated inflorescences formed at least one seed and these inflorescences had 60 seeds per inflorescence on average. However, only 31% or 16.3% of inflorescences formed seeds in the first and third pollination season respectively. There were 23 seeds per inflorescence in the first pollination season and 17 seeds per inflorescence in the third pollination season. The second pollination season had better fertilisation not only in the incompatible crosses but also in the compatible crosses (Table 16). Pollen germination was checked in the

third pollination season. Pollen germination rates were 31.1%, 51.9%, 54.4% and 7.4% for AE_{sp}, AE, GS_{4x} and GS respectively. The reason for the low germination percentage for GS pollen is not known. Pollen germination rates have been found to vary between inflorescences of *Z. aethiopica* 'Childsiana', with values of 45-70% being common (Chi 1990).

Table 17. Average number of seeds in interspecific crosses and self-crosses in the second pollination season

Crosses	WAP	total ovary	% of ovary with seed	seed/ ovary*
AE X EL	4	363	53.0	2.0
	5	263	54.0	1.9
	6	40	37.5	1.0
CHI X EL	4	249	57.8	2.2
	5	440	58.4	2.9
	6	64	65.0	1.4
	7	90	80.0	3.9
CHI _{4x} X EL	4	98	64.3	2.6
	5	40	85.0	2.6
	6	51	62.7	3.1
CHI X CHI	4	112	100.0	5.8
	5	49	81.6	6.7
EL X CHI	4	90	74.4	1.6
	5	27	100.0	1.1
	6	180	75.6	1.7
	7	92	88.0	1.8
EL X CHI _{4x}	4	61	100.0	2.0
	5	66	100.0	2.3
	6	109	76.1	2.0
	9	57	91.2	1.8

*: Based on ovaries with seed development.

In the second pollination season, 94.9% and 100% of inflorescence formed seeds in section I X section I and section II X section II respectively. The percentage of inflorescences forming seeds was also high in section II X section I (95.8%) but was low in section I X section II (65.2%). There were approximately 100 seeds per inflorescence in section I X section I and section II X section II. There were 41 seeds per inflorescence in section I X section II and 69 seeds per inflorescence in section II X section I (Table 16). There were one to four seeds per ovary in AE or CHI X EL but six or seven seeds per ovary for the CHI X CHI. The number of seeds per ovary did not change from four WAP to nine WAP in those incompatible (intersectional) crosses (Table 17).

3.2. Cultures of four and five-week old embryos from compatible crosses

Young embryos from self-pollinations were cultured in order to answer the questions: how early can embryos be rescued *in vitro*? What medium is suitable for embryo development *in vitro*? This information is important for hybrid embryo rescue. A few embryos were available for culture after the first pollination season. The following results were achieved after the second pollination season.

From CHI X CHI, endosperm was soft and was 2.3 X 1.0 mm in size at four WAP. At this stage, the embryo was globular, approximately 0.3 mm in diameter. It was difficult to separate these small embryos from the endosperm under a dissecting microscope, so the embryos embedded in the endosperm were cultured. With these four-week old embryos, 1.0 mg/l BA stimulated embryo germination and plant growth slightly compared to that in the medium without any hormone (Table 18). BA at a concentration of 0.3 mg/l did not have any effect on embryo germination although it stimulated plant growth. However, 3.0 mg/l BA inhibited embryo development. By incorporating 0.3 mg/l NAA with 1.0 mg/l BA, the rate of embryo expansion was decreased and embryo germination was totally inhibited. CH clearly increased the rate of embryo expansion but inhibited embryo germination.

At five WAP in the cross of CHI X CHI, the endosperm was semi-soft and 2.6 x 1.9 mm in size, the embryo was elongating, and close to 1 mm in length. Embryos embedded in endosperm were again cultured. On the medium without any hormone, 92% of embryos expanded and 28% of embryos germinated (Table 18). BA at concentrations of 0.3, 1.0 and 3.0 mg/l greatly stimulated embryo germination and plant growth.

NAA again inhibited embryo expansion, germination and plant growth. CH reduced the rate of embryo germination and affected plant growth.

Table 18. The effects of hormones and casein hydrolysate (CH) on the development of embryos from CHI X CHI *in vitro**

hormone (mg/l)	4 WAP ^a			5 WAP		
	embryo ^b expansion	embryo ^c germination	plant ^d growth	embryo expansion	embryo germination	plant growth
	(%)	(%)		(%)	(%)	
none	8	4	+	92	28	+
0.3 BA	7	4	++	92	66	+++
1.0 BA	10	8	++	92	56	+++
3.0 BA	0	0	0	98	74	+++
1.0 BA + 0.3 NAA	2	0	0	64	16	++
1.0 BA + 400 CH	83	0	0	88	26	++

* 50 embryos embedded in the endosperms were cultured for each treatment.

a: weeks after pollination at the initial culture

b: percentage of embryos which expanded.

c: percentage of embryos which germinated and generated plants

d: growth rate of plants at six weeks after culture.

- 0, embryos did not germinate.
- +, the first leaf yellow or green, but was not open; with no or a little root.
- ++, the first leaf green, opening; good roots.
- +++, the first leaf green, opened; several long (>10 cm) roots (see Fig. 14C).

The development of an embryo of CHI X CHI *in vitro* is shown in Fig. 14. An embryo embedded in the endosperm was cultured at five WAP. The embryo grew out of the endosperm at two weeks after culture. After another two weeks, the cotyledon elongated and turned green. The tip of the first leaf emerged and root formation started. However, the endosperm did not develop. By six weeks, an intact plant had formed with several roots and a green, opening leaf. In contrast, seed on the plant takes approximately 12 weeks to mature. Therefore much more rapid development was achieved using embryo culture.

Embryos of EL X EL were also cultured on a medium with 0.3 mg/l BA. The development of embryos of EL X EL was similar to that of CHI X CHI *in vivo* and *in vitro*. It was difficult to generate plants from four-week old embryos but plant generation from five-week old embryos was much easier.

From self-pollinations, a few embryos (less than 10%) can germinate and generate plants when cultured at four WAP but more than 50% of embryos can generate plants when cultured at five WAP. Hybrid embryo rescue would be executed at four WAP or later. MS medium with 0.3 to 3.0 mg/l BA is a suitable medium for the development of immature embryos *in vitro*.



Fig. 14. Plant generation from a young embryo of CHI *in vitro*

A five-week old embryo embedded within the endosperm was cultured on a medium with 0.3 mg/l BA. Two weeks after culture, the embryo grew out of the endosperm (A). Four weeks after culture, the cotyledon expanded and turned green, the root and leaf were emerging (B). Another two weeks later, an intact plants was generated (C).

3.3. Embryo rescue after the first pollination season

In the first pollination season, intersectional crosses were made on 67 inflorescences (see Appendix 3). Only 135 seeds were harvested. All seeds were used for embryo rescue. Embryos embedded in the endosperms at ages of 4, 5, 6 or 9 weeks were cultured on a medium with 0.3 mg/l BA. Four albino shoots and 15 green plants were generated (Table 19). At 4-6 WAP, embryos were normally very small (< 0.2 mm). These embryos generally did not show further development on the medium. Green plants were generated from four-week old embryos CHI X G and six-week old embryos of CHI X PP (Table 19). These plants, however, were not hybrids because they did not show any hybrid morphological characters; their flowers were similar to CHI seedlings, there was no evidence for paternal DNA bands when they were tested by DNA probes (Section 3.1 of Chapter 9). In the CHI X PP cross, one albino shoot was also generated from a six-week old embryo. At 13 WAP, 14 ovaries from one inflorescence were harvested. Seven embryos about 1 mm in length were dissected and cultured on the medium. Three of these embryos generated albino shoots (Table 19). The hybridity of these albino shoots were confirmed by DNA probing (Section 3.1 of Chapter 9).

Table 19. Embryo rescue after the first pollination season *

crosses	WAP	embryos cultured	developed embryos	green plants	albino shoots
GS X CHI	5	6	0	0	0
GS X CHI	6	2	0	0	0
GS _{4x} X CHI	4	28	1	0	0
GS _{4x} X CHI	6	11	0	0	0
GS _{4x} X AE	4	2	0	0	0
BM _{4x} X CHI	4	12	0	0	0
AE X G	4	6	0	0	0
AE X G	6	1	0	0	0
CHI X PP	4	11	0	0	0
CHI X PP	6	13	8	7	1
CHI X G	4	36	12	8	0
G X CHI	13	7	3	0	3
total		135	24	15	4

* Medium supplemented with 0.3 mg/l BA and 3% sucrose.

3.4. Embryo rescue after the second pollination season

Z. aethiopica (AE) X *Z. elliotiana* (EL)

At four WAP, the endosperm of AE X EL was about 2.5 x 1.5 mm in size and very soft. Most were not distinguishable from those of AE X AE. The embryos were globular (<0.2mm). At five WAP, many endosperms of AE X EL were watery and transparent (Fig. 15), and could be distinguished from the endosperm of AE x AE. The embryos of AE X EL at five WAP were still globular in shape while those of AE X AE had elongated and were approximately 1 mm in length. At six WAP, more of the endosperms appeared watery and transparent, and there was still variability in size. Embryos did not show any elongation although they may have increased a little in size.

Table 20. Embryo rescue after the second pollination season, from AE X EL

WAP	BA (mg/l)	embryos cultured	developed		green plants	albino	
			No.	(%)		No.	(%)
4	0.3	124	39	31.5	9	18	14.5
	1.0	70	3	4.3	0	0	
	3.0	62	0		0	0	
5	0.3	131	32	24.4	0	27	23.5
	1.0	44	21	47.7	0	20	45.5
6	0.3	15	10	66.7	0	10	66.7
total		446	105	23.5	9	75	16.8

Embryos of different ages (4, 5, or 6 WAP) developed similarly *in vitro*. Some of the embryos enlarged and grew out of the endosperm, with or without expansion of the cotyledon (Fig. 16). Most of these embryos generated multiple albino shoots when they were subcultured to a medium with 0.3 or 1.0 mg/l BA (Fig. 17). Some of the endosperms did not show any growth in culture but other endosperms generated a large amount of callus which was white, soft, and watery (Fig. 16), without any evidence of regeneration.

From AE X EL, 446 embryos embedded in endosperm were cultured at 4, 5 or 6 WAP on medium with 0.3, 1.0 or 3.0 mg/l BA (Table 20). Of all these cultured embryos, 23.5% showed some development, 16.8% generated albino shoots and 2.0% generated green plants. These green plants, however, were later shown to have arisen from self-pollination. With four-week old embryos, the best development was found on a medium with 0.3 mg/l BA (Table 20). As the age of the embryos increased, the percentage of embryos which generated albino shoots also increased (Table 20, Fig. 18).

***Z. aethiopica* 'Childsiana' (CHI) X *Z. elliotiana* (EL)**

The development of endosperm and embryo from CHI X EL was approximately a week slower than that from AE X EL. Otherwise the progress of development was similar. The embryos also generated multiple albino shoots and some of the endosperms produced a white soft callus in culture.

From CHI X EL, 751 embryos embedded in endosperm were cultured at 4, 5, 7, or 10 WAP (Table 21). Of these cultured embryos, 19.0% showed some embryo development and 13.0% generated albino shoots. Twenty-seven green plants were also generated. However, all these green plants were later found to be the results of self-pollinations (Chapter 9). With five-week old embryos, CH stimulated the embryo enlargement but did not increase the proportion of embryos which generated albino shoots. With seven-week old embryos, CH had little effect on embryo enlargement or albino shoot formation. On a medium with 0.3 mg/l BA, the proportion of embryos forming albino shoots generally increased as the age of the embryos increased from four WAP to ten WAP (Table 21, Fig. 18).

At ten WAP, 38 ovaries were harvested from one inflorescence. In these ovaries, most ovules were empty, i.e., they had thick outer-integuments but the endosperm had aborted. Endosperm was found in 90 ovules. In most of these ovules, the endosperm was generally soft and watery and contained a small round embryo. Six ovules, however, contained solid endosperms and elongated embryos. The size of the embryos was similar to self-pollinated embryos at seven WAP. The six green plants (Table 21) which originated from these six embryos were later shown not to be hybrids.

Table 21. Embryo rescue after the second pollination season, from CHI X EL

WAP	BA (mg/l)	embryos cultured	developed embryos		green plants	albino shoots	
			No.	(%)		No.	(%)
4	0.3	82	5	6.1	1	3	3.7
	1.0	45	0		0	0	
	3.0	60	3	5.0	0	3	5.0
5	0.3	162	32	19.8	5	27	16.7
	0.3 + (500 CH)	77	30	39.0	8	9	11.7
	1.0	50	4	8.0	0	2	4.0
7	0.3	105	13	12.4	4	8	7.6
	0.3 + (500 CH)	80	11	13.8	3	7	8.8
10	0.3	90	45	50.0	6	39	43.4
total		751	143	19.0	27	98	13.0

***Z. aethiopica* 'Childslana' (CHI_{4x}) X *Z. elliotiana* (EL)**

Tetraploid plants of CHI (CHI_{4x}) which were induced by colchicine treatment of germinating seeds (Chapter 5) were used as the maternal parent. There were no differences in the development of embryos or endosperms of CHI_{4x} X EL compared with CHI X EL. Also, cultured embryos from these two crosses responded similarly. From CHI_{4x} X EL, 171 embryos embedded in endosperm were cultured at 4, 5, or 6 WAP on a medium with 0.3 mg/l BA (Table 22). Of these cultured embryos, 15.2% showed some increase in embryo size and 7.0% generated albino shoots. The proportion of embryos forming albino shoots generally increased as the age (WAP) of the embryos increased (Fig. 18). One green plant resulting from self-pollination was also produced. Some endosperms produced a white soft callus in culture,

Table 22. Embryo rescue after the second pollination season, from CHI_{4x} X EL

WAP	BA (mg/l)	embryos cultured	developed embryos		green plants	albino shoots	
			No.	(%)		No.	(%)
4	0.3	72	4	5.6	0	3	4.2
5	0.3	53	7	13.2	0	3	5.7
6	0.3	46	15	32.6	1	6	13.0
total		171	26	15.2	1	12	7.0

***Z. elliotiana* (EL) X *Z. aethiopica* 'Childsiana' (CHI) (2x,4x)**

The development of embryos and endosperm were similar in EL X CHI and EL X CHI_{4x}. At four WAP, the endosperm size was about 0.8 x 1.9 mm. Endosperms were soft and watery and embryos were small (<0.2 mm). The endosperm size increased to about 2 x 2.8 mm at five WAP and about 2.8 x 3.4 at six WAP. At six WAP, endosperms were still soft and embryos were about 0.5 mm in length.

From EL X CHI, 514 embryos embedded in endosperm were cultured on media with 0.3 or 1.0 mg/l BA at 4, 5, 6 or 7 WAP. After four weeks in culture, the embryos still remained within the endosperm. At this time embryos were dissected out; some were subcultured on a medium with 1.0 mg/l BA, while others were transplanted into nurse endosperm (endosperm from a compatible cross) and cultured on a medium with 1.0 mg/l BA. At the time of dissection, 45.1% had increased in size (up to 1.5 mm long) during the four week culture period. The embryos placed directly on a medium with 1 mg/l BA and the embryos placed in nurse endosperm enlarged, formed callus but none of the embryos developed shoots.

The embryos of EL X CHI_{4x} were treated similarly and gave similar results. From EL X CHI_{4x}, 530 embryos were cultured between four and nine WAP. Of these embryos, 37% showed an increase in size but no shoots formed.

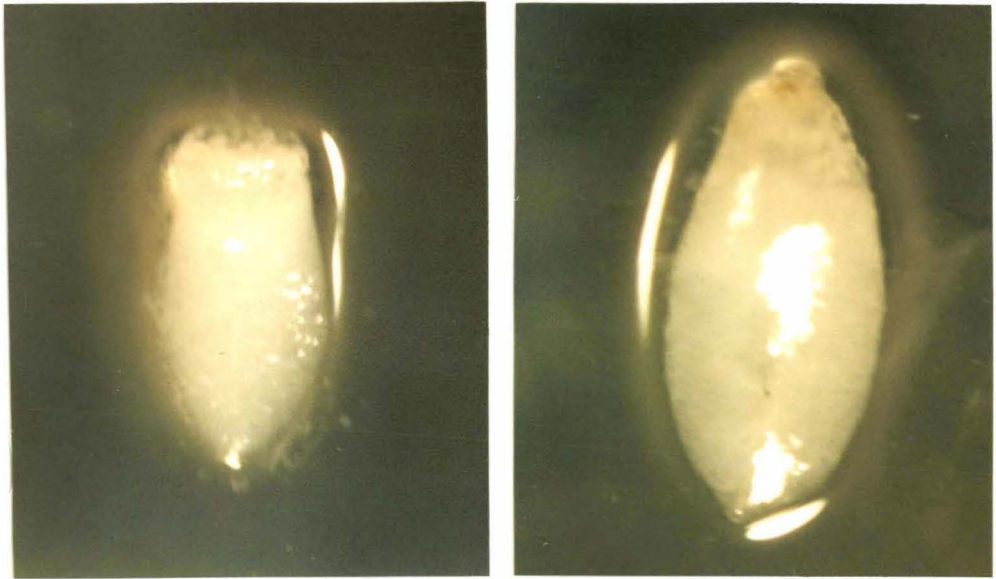


Fig. 15. Degenerating endosperms from incompatible cross (AE X EL) at six WAP

These endosperms were watery and transparent, unlike the endosperm of compatible cross in Fig. 14.

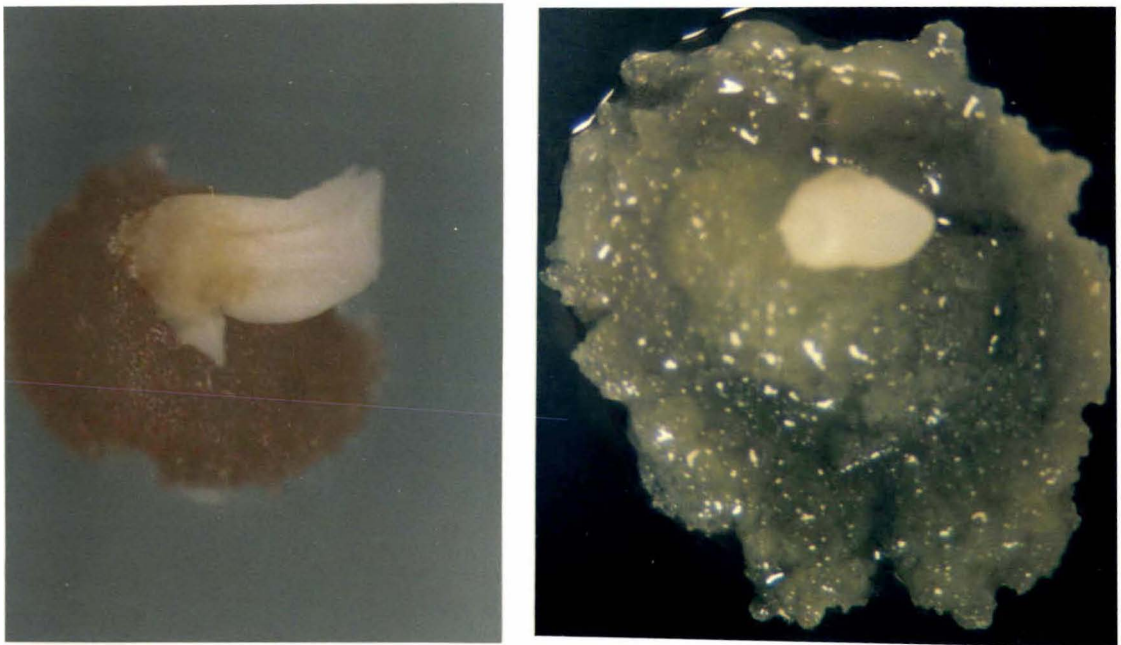


Fig. 16. Development of embryos and endosperms from AE X EL *in vitro*

After three weeks on a medium with 0.3 mg/l BA, the embryos grew out of the endosperms. Embryos may start germination with expansion of the cotyledon (left) or endosperm may generate soft, white callus (right).

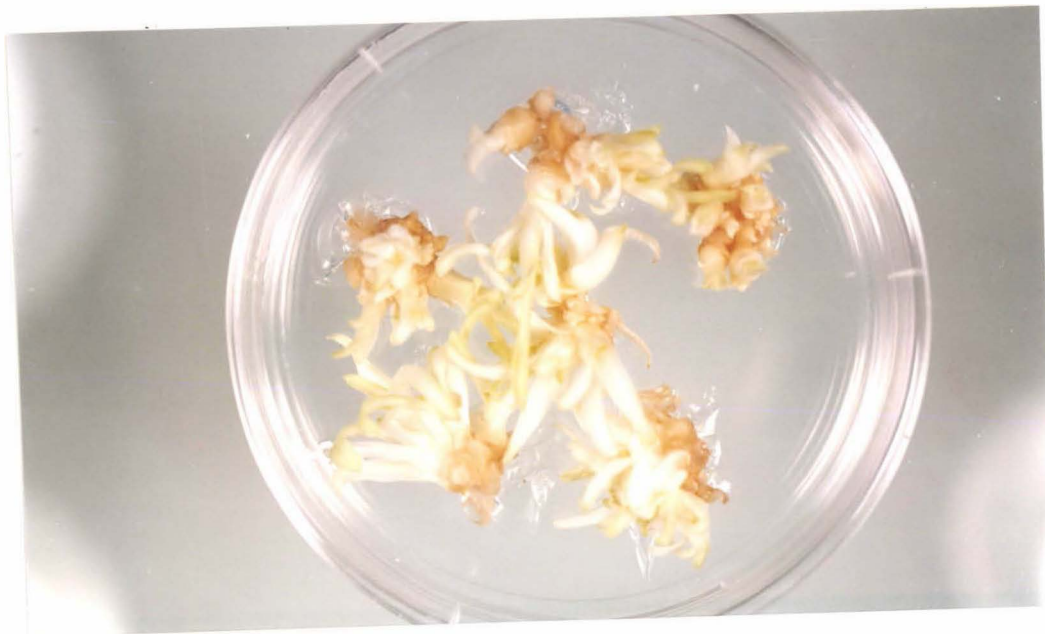


Fig. 17. Albino shoots generated from incompatible crosses (AE X EL) *in vitro*
Embryos (as shown in Fig. 16) generated multiple shoots in subcultures on a medium with 1.0 mg/l BA.

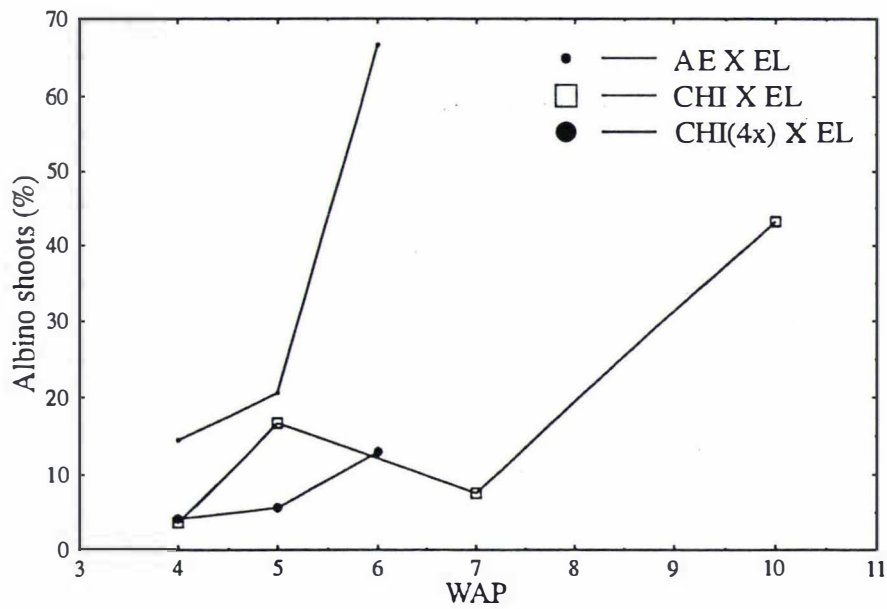


Fig. 18. Percentage of embryos generating albino shoots plotted against embryo age (WAP)

Embryos were cultured on MS medium with 0.3 mg/l BA at different WAP.

3.5. Embryo rescue after the third pollination season

Although 118 inflorescences were pollinated with pollen from plants in another section (see Appendix 3), only 136 seeds were available for embryo rescue because of poor fertilisation. Embryos embedded in endosperm from these 136 seeds were cultured on a medium with 0.3 mg/l BA. The results of embryo rescue at this time is summarised in Table 23. Albino shoots were formed on seven embryos. All seven albino shoots were formed on nine- or ten-week old embryos whereas five- to seven-week old embryos did not produce any shoots. No green plants were formed. When *Z. aethiopica* was used as the maternal parent, soft white callus developed from endosperms in culture. However, no callus developed from endosperm in culture when plants from summer-flowering species were used as the maternal parent.

Table 23. Embryo rescue after the third pollination season

crosses	WAP	embryos cultured	developed embryos	green plants	albino shoots
RE X CHI	5	21	0	0	0
RG X CHI	7	8	1	0	0
AE X PPK	7	14	0	0	0
AE X RE	7	20	0	0	0
CHI X G	9	7	1	0	0
BG X AE _{sp}	9	40	4	0	3
PS X AE _{sp}	9	10	2	0	2
G X AE _{sp}	10	16	4	0	2
Total		136	12	0	7

3.6. The ultra-structure of plastids

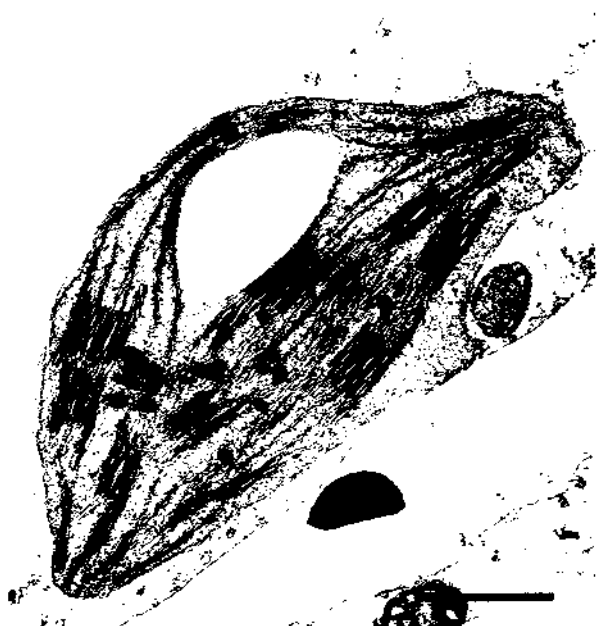
To determine the plastid structure in the leaves of albino hybrids in the dark and in the light, shoots of albino hybrids and green genotypes were grown in the dark and in the light (Section 2.5). After five weeks in the dark, the plastids of green genotypes formed a typical prolamellar body (Fig. 19.A), whereas this prolamellar body did not form in plastids of the albino hybrids (Fig. 19.B). Large starch grains were formed in the plastids of both green genotypes and albino hybrids. For leaves grown in the light, plastids developed into chloroplasts with stromal thylakoid membrane and grana in green genotypes (Fig. 19.C), whereas the plastids in albino hybrids developed only restricted internal-membrane but no grana (Fig. 19.D). Large starch grains were found in green genotypes only. The ultra-structure of plastids was similar in three green genotypes examined and similar in albino hybrids examined from two crosses. The differences of plastid structure between parents and albino hybrids indicated that the plastid development in the albino hybrids was abnormal not only in the light but also in the dark.



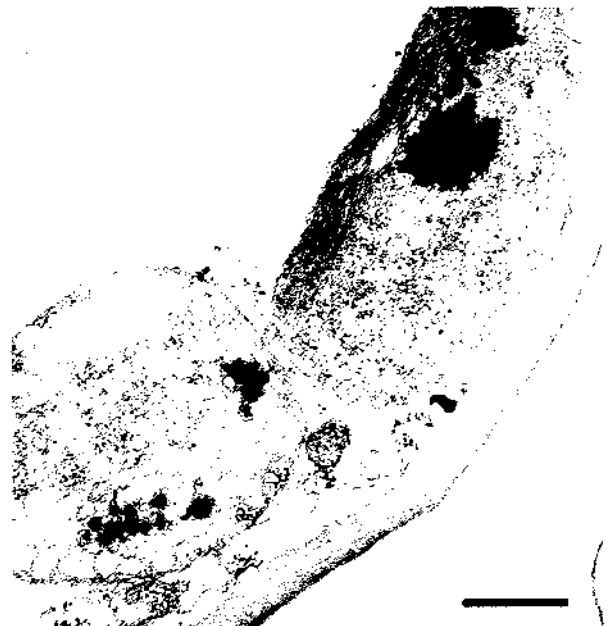
A



B



C



D

Fig. 19. Ultra-structure of plastids of green genotypes and albino hybrids grown in the dark and in the light

(A) and (C): green genotype

(B) and (D): albino hybrids

(A) and (B): in the dark

(C) and (D): in the light

Bar: 1 μm

4. Discussion

Fertilisation

In compatible crosses, fertilised ovules developed into mature seeds with normal endosperm and embryos. In incompatible crosses, fertilised ovules developed slowly for eight to ten weeks into abnormal seeds containing degenerating endosperm and globular embryos. The number of seeds per ovary were lower in incompatible crosses AE or CHI X EL (1 to 4) than in compatible crosses CHI X CHI (6 or 7) but the number of seeds per ovary in the incompatible crosses did not decrease from four to nine WAP (Table 17). This indicated that the lower number of seeds per ovary was caused by poor fertilisation in the incompatible crosses. The poor fertilisation could be caused by prefertilisation incompatibility.

The number of seeds per inflorescence was lower in the first and third pollination seasons than in the second pollination season. Poor fertilisation occurred in these two pollination seasons, viz April to June 1990 and February to May 1991. Pollen used in the third pollination season was tested for germination *in vitro*. The germination rate measured (between 7.4% - 54.4%) was lower than the 70% germination reported by Chi (1990). It is not known how the pollen germination percentage measured *in vitro* related to pollen germination *in vivo* under greenhouse conditions. Low pollen germination may be a reason for poor fertilisation but as large amount of pollen was applied to the stigmas, it is expected that even if pollen germination was poor, fertilisation would still have occurred. Low receptivity of the stigmas may be another reason for poor fertilisation. However, it is not known which factor affected fertilisation in the present study.

Postfertilisation incompatibility

In this study an attempt was made to study the effect of genotype, cross direction, and ploidy level on interspecific hybrid formation in *Zantedeschia*. Unfortunately these attempts were affected by poor fertilisation in the first and the third pollination seasons. In these two pollination seasons, a number of genotypes and some genotypes with two ploidy levels were used in reciprocal crosses. The poor fertilisation made a number of comparisons impossible or less effective. In the second pollination season, even though fertilisation was good, fewer genotypes were used. Some comparisons can be made based mainly on the result from the second pollination. The assessment on

crossability is based on the production of albino shoots because no green hybrids were rescued from any cross between the two sections.

The results from the second pollination season showed that the cross direction affected the crossability. When AE, CHI and CHI_{4x} were used as maternal parent, 16.8%, 13.0% and 7.0% of embryos generated albino shoots (Tables 20, 21, 22). No albino shoots were generated when EL was used as the maternal parent. Endosperms in the former direction formed a large amount of white soft callus *in vitro*. Endosperms in the latter direction did not form any callus. The effects of cross direction on crossability have been observed in a number of interspecific hybridisations (Price & Stutz 1974, Mok *et al.* 1978, Rabakoarihanta *et al.* 1979, Shii *et al.* 1982, Pandey *et al.* 1987, Nkongolo *et al.* 1991). For example, in the crosses between bread wheat and triticale, the use of triticale as the female has given significantly fewer embryos, but these embryos were symmetrically shaped and normal. The reciprocal cross, however, has given a good seed set, but embryo necrosis resulted in rapid death *in vivo* or non-regeneration *in vitro* (Nkongolo *et al.* 1991). The change of crossing direction can change the genome ratio between the two parents in the endosperm, the cytoplasmic genes in the endosperm and embryo, and the interaction between the embryo sac and the maternal tissue. These changes may have effects on the hybrid embryo development.

From the crosses with CHI or AE as maternal parent, a number of green plants were generated by self-pollinations. It should be noted that the inflorescences were not emasculated. New (1964) reported that *Z. aethiopica* stigmas lost their pollen receptivity when pollen was released from the same inflorescence. In the present experiments, it is possible that a small amount *Z. aethiopica* pollen was inadvertently spread during the repeat pollinations. There were no green plants generated when EL was used as the maternal parent but in this case the inflorescences were emasculated before any pollen was visible.

From CHI x EL (Table 21) and CHI_{4x} X EL (Table 22), 13.0% of 751 embryos and 7.0% of 171 embryos respectively, generated albino shoots *in vitro* but no green hybrids were generated from either cross. The development of endosperm and embryos in these two crosses was very similar. No shoots developed from more than 1000 cultured embryos of EL X CHI or EL X CHI_{4x}. Fifty-three embryos obtained from crosses involving tetraploid GS and tetraploid BM as the maternal parent were cultured (Table 19). These embryos were globular at the time of culture and did not

generate any shoots. It appeared that the use of tetraploid plants as one parent did not improve the crossability between the two sections of *Zantedeschia*. CHI_{4x} plants used may be tetraploid/diploid chimeras. Further study using pure tetraploid plants of CHI is necessary in order to confirm the effect of tetraploid CHI on the crossability. The change of ploidy level of one parent changes the endosperm balance number (EBN) (Johnston *et al.* 1980) ratio between the two parents in the hybrid endosperm. The EBN ratio sometimes plays an important role in hybrid embryo development. Manipulation of EBN has overcome the interspecific incompatibility in some genera, such as *Solanum* (Johnston *et al.* 1980), *Trifolium* (Parrott & Smith 1986) and *Medicago* (McCoy & Smith 1984) although it has not overcome the interspecific incompatibility in *Cyphomandra* (Pringle & Murray 1991). Matching EBN is a necessary but not a sufficient condition for a successful cross. Successful crosses may be prevented by other barriers unrelated to EBN even if the EBN is matched. In hybridisation between the two sections of *Zantedeschia*, barriers unrelated to EBN, such as hybrid albinism, are present.

In an incompatible cross, endosperm and embryo begin development after fertilisation. At a later stage the endosperm may degenerate and the embryo must be rescued soon after this stage. A suitable time for embryo rescue always needs to be determined experimentally. Embryos embedded in the endosperms were cultured at 4, 5, 6, 7, and 10 WAP. The percentage of embryos that generated albino shoots generally increased from four WAP to ten WAP with the exception of seven WAP for CHI X EL (Fig. 18). At four WAP, embryos might be too small to rescue. By six WAP some embryos might have elongated. From six WAP, some seeds had a badly degenerated endosperm which was watery and transparent. At ten WAP, some seeds were empty because of the complete degeneration of the endosperm within the thick seed coat. No attempt was made to rescue embryos from seeds with badly degenerated endosperm. At four or five WAP, it was not possible to grade the endosperm in this way and almost all seeds were used in culture without selection. It is possible that the better embryo rescue results from six WAP are because of this screening. The suitable time for rescuing embryos from incompatible *Zantedeschia* crosses appeared to be between six to ten WAP. This agrees with the previous study (Chi 1990). The age of hybrid embryo has been found to be important for embryo rescue in *Brassica juncea* X *B. hirta* (Mohapatra & Bajaj 1987).

Hybrid albinism

Albino hybrids have previously been generated from interspecific hybridisation in many genera, including *Oenothera* (Kirk & Tilney-Bassett 1978), *Trifolium* (Hovin 1962a, Pandey *et al.* 1987, Przywara *et al.* 1989) and *Impatiens* (Arisumi 1985). The albinism may be unidirectional (Hovin 1962a, Pandey *et al.* 1987) or bidirectional (Przywara *et al.* 1989) and the percentage of green plants varied according to the plant species. The hybrid albinism is thought to be caused by incompatibility between the plastome (plastid genome) and the genome (nuclear genome) (Kirk & Tilney-Bassett 1978). In *Oenothera*, five types of plastome and six types of genome were classified. Certain plastome was only compatible with particular types of genome. Plastome type IV was compatible with the widest range of genomes, and genome AB was compatible with the widest range of plastomes (Stubbe 1964). If plastids are inherited maternally, a plastid type which may be more compatible with the hybrid nucleus can be chosen by changing the cross direction. A genome with improved compatibility to a certain type of plastome can also^{be} achieved by selecting different paternal genotypes.

From crosses within section II of *Zantedeschia*, a majority of hybrids are green and minority of hybrids are albino, chimeric or virescent (New & Paris 1967). From 'Chromatella' (section II) X 'Childsiana' (Section I), only albino hybrids were produced from three embryos even though many embryos were cultured *in vitro* (Chi 1990). It was postulated that green or partially green hybrids might have been obtained if more hybrids had been rescued from different crosses. However, only albino hybrids were obtained even if 182 embryos generated shoots after approximately 2500 embryos were cultured in the present study (Tables 18, 20, 21, 22, 23). The crosses which produced albino shoots involved three genotypes (AE, AE_{sp}, CHI) of *Z. aethiopica* in section I, one species *Z. elliotiana* and four hybrid cultivars, G, BG, PS and PP in section II. The albinism between the two sections was bidirectional because albino hybrids were produced in crosses with plants of either section as maternal parent. This bidirectional albinism has been supported by the presence of plastids from both parents in several albino hybrids (Section 3.2 of Chapter 9).

Plastids in *Zantedeschia* albino hybrids had no prolamellar body in the dark nor grana in the light although plastids of the parents had a typical prolamellar body in the dark and many grana in the light (Fig. 19). However, even in the albino leaves, large starch grains formed in plastids in the dark. This demonstrates that at least the enzymes for starch synthesis were active in the albino plastids. Przywara *et al.* (1989) noted the

absence of internal membrane in plastids in the albino sectors of *Trifolium repens* X *T. hybridum* hybrids. Mascia and Robertson (1978) also noted the absence of a prolamellar body in two yellow mutants of maize. In *Zantedeschia* albino hybrids, the absence of prolamellar body and grana indicated that the development of plastids was abnormal not only in the light but also in the dark. The formation of the prolamellar body and thylakoid membranes is the result of the expression of many genes which were in some way blocked in *Zantedeschia* albino hybrids. Many albino plants have been produced from anther culture of cereals and the albinism is mainly controlled by a few nuclear genes (Agache *et al.* 1989, Tuveesson *et al.* 1989, Larsen *et al.* 1991). In most cases, however, the genetic control mechanisms which control albinism are still unclear.

It was evident from this study that incompatibility between the two sections of *Zantedeschia* occurred at three different stages. Firstly, prefertilisation incompatibility reduced the number of fertilised ovules developing in the ovaries. However, many eggs were still fertilised and formed endosperms and embryos, so the prefertilisation barrier was only partial. Secondly, postfertilisation incompatibility caused poor embryo development and endosperm degeneration. Many endosperms were watery and transparent indicating poor starch deposition (Chi 1990). The development of most embryos was restricted in the globular stage. The barrier to embryo development was overcome in many cases by using embryo culture. About two hundred albino hybrids were rescued from several crosses (Tables 18, 20, 21, 22, 23). It is concluded that plastome/genome incompatibility, at the hybrid plant development stage, is the principal factor restricting intersectional hybridisation of *Zantedeschia*.

CHAPTER 7. Testing *Z. odorata* as a bridge for gene transfer between the two sections of *Zantedeschia*

1. Introduction

In the previous Chapter, hybridisation between the two sections of *Zantedeschia* produced only albino hybrids. These hybrids survived only on artificial medium. The hybrid albinism could not be overcome by using a range of genotypes from both sections, changing ploidy level of the parents or via *in vitro* culture technique.

In some wide hybridisations of plants, bridge lines have been used for gene transfer between two species which are highly incompatible. A natural species *Nicotiana sylvestris* has bridged disease resistant genes from *N. repanda* to *N. tabacum* (Burk 1967). Artificial bridge lines have been used for gene transfer between species in the genus *Lycopersicon* (Poysa 1990) and between the genera *Lycopersicon* and *Solanum* (Deverna *et al.* 1990). The basic principle for bridge hybridisation is: if species A and C are highly incompatible and an intermediary species B has partial compatibility with both A and C, crosses may be made firstly between A and B and between B and C. Hybridisation may then be possible between AB hybrids and BC hybrids, between AB hybrids and C or between BC hybrids and A. Chromosome doubling of hybrids or one parent may also be used to produce a sesquidiploid line which may transfer one chromosome or a partial genome from one species to another (Burk 1967, Deverna *et al.* 1990). Chromosome doubling may also be necessary for restoring hybrid fertility.

In the middle stage of the current study, a newly-discovered species *Z. odorata* was kindly supplied by Perry (National Botanic Gardens, South Africa). *Z. odorata* has some morphological characters from each section and appears to be intermediate between the two sections (Perry 1989, Chapter 2). The results of chromosome karyotype analysis (Chapter 4) and RFLP analysis (Chapter 8) have also demonstrated an intermediary position of *Z. odorata*. We, therefore, hoped *Z. odorata* may act as a bridge to facilitate gene transfer between the two sections. However, Perry (1989) reported that from more than 100 plants in cultivation over five years only one flower was produced. We used gibberellic acid (GA) application in an attempt to increase flower production of *Z. odorata* since GA increases flower production of plants in

section II of *Zantedeschia* (Corr & Widmer 1987, Funnell *et al.* 1988). This study attempted the hybridisations between *Z. odorata* and section I, and between *Z. odorata* and section II.

2. Material and methods

Plant material. The following plant material was used. *Z. odorata* (OD) was obtained from Perry (collection No: *Perry & Snijman 2147*). Three genotypes (AE, AEsp, CHI) of *Z. aethiopica* (section I), *Z. rehmannii* (RE) and *Z. 'Chromatella'* (CHR) were the same as described in Chapters 4, 5, and 6. One diploid-tetraploid chimera plant of 'Childsiana' (CHI-T58) was derived from colchicine-treated seedlings (see Section 3.4 of Chapter 5).

Embryo culture. The pollination and embryo rescue methods were similar to those described in Chapter 6. Emasculation was made on OD by removing the male zone. Pollinations were executed with fresh pollen between 15th July and 4th September, 1991 in a greenhouse. The number of inflorescences pollinated for each cross are listed in Appendix 4. Isolated embryos or embryos embedded in endosperms from five to eleven weeks after pollination (WAP) were cultured on MS medium with 0.3 mg/l BA. The medium for embryo culture was the same as described in Chapter 5.

Seed collection. Fruit were harvested at 12 WAP and stored in the laboratory for approximately two weeks to soften the ovary wall. Seeds were collected by washing the ovary wall away and they were then air dried for one week.

Germination test. Air-dried seeds were either stored in room temperature or were sterilised with bleach (as described in Chapter 5). Sterile seeds were sown in properly wetted, autoclaved peat:pumice (50:50) mix in 200 ml jars and germinated at 21 °C. Germinated seeds were transferred into a greenhouse after six weeks. After 2 months storage, seeds of OD (self-pollinated) and AE X CHI were immersed in 6, 25 or 100 mg/l GA₃ solution, or water (as a control), for three days and then sterilised and sown in the peat-pumice mix to germinate.

Chromosome counts were made for hybrids between *Z. odorata* (maternal parent) and CHI-T58 to determine the ploidy level of hybrids. The method of chromosome preparation was the same as described in Chapter 5.

3. Results

3.1. Reproductive characters of *Z. odorata*

Six tubers of *Z. odorata* (OD) were planted in May 1990 without GA treatment and did not produce any flowers after nine months even though many leaves formed. Two of these plants were sprayed with 50 mg/l GA₃ on the leaves in February 1991 and flowered seven months later. The other four plants which were not sprayed with GA had not flowered by January 1992. Thirteen dry-stored tubers were dipped in 50 mg/l GA₄₊₇ and planted in March 1991. The resulting plants flowered from the main buds at approximately four-months old when three or four leaves formed. Flowers later arose from the second or third axillary buds.

In OD, male flowers on the upper part of the spadix started to release pollen approximately two days after the spathe fully opened. Another one to three days later, pollen was also released from the 'staminodes' interspersed between the female flowers. The male zone was cut off from four OD inflorescences. One of these inflorescences was pollinated with OD pollen at the time of the spathe starting open. The remaining three inflorescences were pollinated with OD pollen either at the time the spathe fully opened, three days or six days after the spathe fully opened. Good seed set was achieved from all these four inflorescences. Thus the stigmas of OD were receptive to pollen at least from the time of spathe opening (approximately two days before the spathe fully opened) to six days after full spathe opening. Emasculation is therefore necessary to prevent self-pollination because pollen is released before the stigmas lose their pollen receptivity.

The *in vivo* development of endosperms and embryos of OD was found to be similar to that of other *Zantedeschia* species described previously. The endosperm was soft at five WAP, semi-soft at six or seven WAP and subsequently hardening. At nine WAP, the endosperm was hard and almost fully-matured. The embryos elongated to approximately 0.5 mm in size at five WAP. The length of the embryos reached to 1.6 mm, 1.9 mm, 2.8 mm at six, seven and nine WAP respectively (Table 24). A fully-matured embryo was about 3.0 mm long. On average, three seeds per ovary were formed (Table 25).

MS medium with 0.3 mg/l BA was the best medium for the *in vitro* development of embryos of section I and section II (Chapter 6). Embryos of AE and EL generally gave 100% germination when they were cultured at six and seven WAP and produced normal seedlings. On the same medium, however, OD embryos only showed 76.5% and 61.8% germination when they were cultured at six and seven WAP (Table 24). The growth of seedlings was slow and many plants died on the medium. Furthermore, no embryos germinated when they were cultured at nine WAP (Table 24). After two month storage, however, dissected embryos of OD seeds achieved 100% germination on the same medium.

Table 24. Development of OD embryos (self-pollinated) *in vivo* and *in vitro*

WAP	embryo size (mm)	embryos cultured	plants generated	
			N.	%
6	1.6	17	13	76.5
7	1.9	61	38	61.8
9	2.8	65	0	
*	3.0	30	30	100

*: Seeds were collected at 12 WAP and stored at room temperature for two months before the embryos were dissected and cultured.

3.2. Hybrid embryo development and *in vitro* culture

***Z. aethiopica* (AE) X *Z. odorata* (OD)**

After fertilisation, the development of endosperms and embryos was quite normal in AE X OD. At six WAP, 80% of embryos reached 1.0-1.2 mm in length and the remaining embryos were approximately 0.8 mm long. The endosperms associated with the smaller-sized embryos were also poorly developed. The average embryo size was 1.7 mm, 2.2 mm, 2.6 mm long at seven, eight, and nine WAP respectively. The endosperm varied from soft, semi-soft to hard. Most embryos and endosperms did not show much sign of incompatibility. An average of four seeds per ovary was formed (Table 25). A number of mature seeds with well-developed embryos was harvested.

Table 25. Number of seeds per ovary of different crosses

Cross	ovary	seed	seed/ovary
AE X OD	394	1607	4.1
AEsp X OD	15	77	5.1
CHI X OD	96	308	3.2
OD X AE	41	188	4.6
OD X CHI	93	136	3.7
RE X OD	22	24	1.1
CHR X OD	229	480	2.1
OD X CHR	254	423	1.7
CHI X CHI	16	62	3.9
OD X OD	282	887	3.1
CHR X CHR	27	77	2.9

The development of seven-week old embryos *in vitro* was closely investigated. The haustorial tip of the cotyledon did not expand. The rest of the cotyledon expanded rapidly and turned pale green. The expansion of the cotyledon was visible from two days after culture and fully expanded after about 12 days. Then the first leaf emerged and roots formed. After the embryos germinated, young seedlings of AE X OD were pale green compared to seedlings of CHI X CHI on the same medium (Fig. 20).

Seventy-five embryos embedded in the endosperms were cultured at five WAP. Of these embryos, 90% which increased in size were dissected out from their endosperms and subcultured. Pale green plants were produced from 41% of the embryos. Of embryos cultured at six, seven, eight and nine WAP, 62.0%, 86.4%, 87.0% and 74.2% generated pale green plants (Table 26). All these plants grew well on the medium and more than 90% survived in a greenhouse. The hybridity of 18 plants tested was confirmed by Southern hybridisation using an rDNA probe (Chapter 9).

Table 26. Development and *in vitro* culture of embryos of crosses between AE and OD ^a

WAP	embryo size(mm) <i>in vivo</i>	total embryo cultured	developed embryos ^b N. %		generated plants N. %	
AE X OD						
5	0.8	75 ^c	67	89.3	31	41.1
6	1.1	71	71	100	44	62.0
7	1.7	125	124	99.2	108	86.4
8	2.2	58	58	100	51	87.0
9	2.6	31	31	100	23	74.2
OD X AE						
7	0.6	30 ^c 30	18	30.0	2	3.3
9	1.7	62	1	1.6	0	
11	2.5	68	2	2.9	1	1.5

a: MS medium with 0.3 mg/l BA was used.

b: Embryos which showed an increase in size after four weeks in culture.

c: Embryos embedded within endosperms were cultured, otherwise isolated embryos were cultured.

These hybrid plants were heavily shaded in a greenhouse to protect them from photo-bleaching in strong sun light. During the first two weeks in the greenhouse, some of the leaves which formed in the culture container were variegated with green, pale green or white sectors. Subsequently all newly-emerged young leaves were yellow but became greener as leaves aged. The greening of the leaf veins was faster than the rest of the leaf tissue (Fig. 21). However, even old leaves were not as green as leaves of parents when the plants were less than five-months old. By five months, old leaves were as green as parents but newly-emerged young leaves were either yellow or green. Some hybrid plants flowered after they were grown in a greenhouse for nine months. These hybrids were fertile as many seeds set in back-crosses. At this stage, the leaves of hybrids were as green as the leaves of the parents.

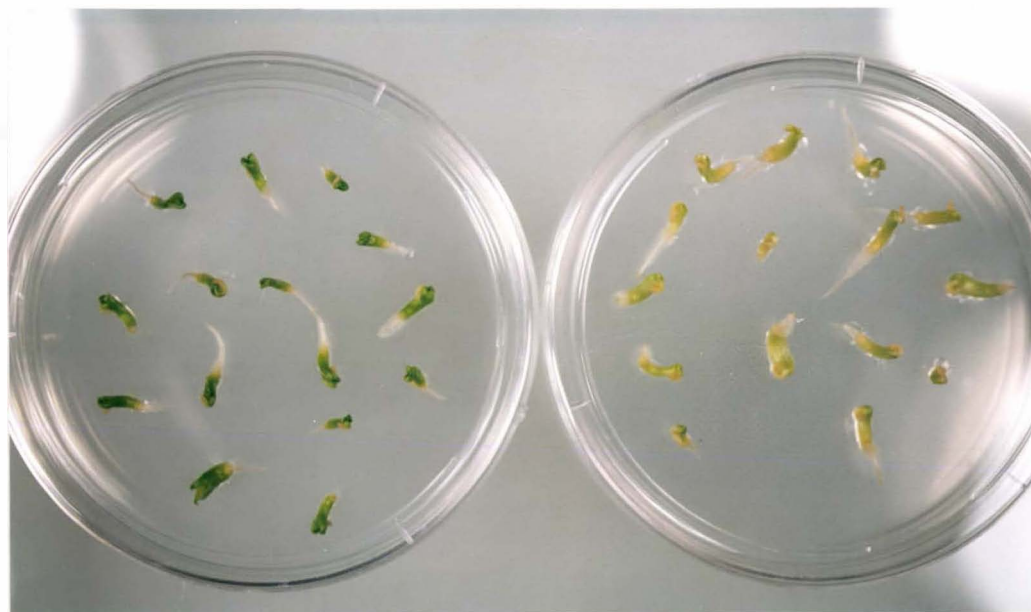


Fig. 20. Green seedlings of CHI X CHI (left) and pale green seedlings of CHI X OD (right)
Embryos of both crosses were excised and cultured at ten WAP. Photograph was taken at two weeks after culture. The seedlings of AE or AEsp X OD were also pale green, they are not shown here.



Fig. 21. A virescent hybrid of AE X OD

Young leaves were yellow or pale green and older leaves were green. The veins were greening faster than the rest of leaf tissue. Other seedlings of AE X OD and OD X AE were similar to this plant.

***Z. aethiopica* 'Childsiana' (CHI) X *Z. odorata* (OD)**

The endosperms and embryos from CHI X OD developed normally and were much like those from AE X OD. Most ovules did not show much sign of incompatibility. The embryo development of CHI X OD (Table 27) was approximately one week slower than that of AE X OD (Table 26) by comparing the embryo sizes. This slow development was also observed by comparing CHI X EL with AE X EL (see Section 3.4 of Chapter 6). In CHI X OD, a high percentage (76%, 100%) of embryos generated plants after they were cultured at eight and ten WAP (Table 27). The seedlings were pale green on the medium (Fig. 20) and virescent in a greenhouse. Greening was achieved as leaves aged. The greening was uniform over the leaf tissue. The hybridity of seven plants tested was confirmed by Southern hybridisation (Chapter 9).

Table 27. Development and *in vitro* culture of embryos of crosses between CHI and OD ^a

WAP	embryo size(mm) <i>in vivo</i>	total embryo cultured	developed embryos ^b		generated plants	
			N.	%	N.	%
CHI X OD						
5	0.6	17 ^c	15	88.2	10	58.8
6	0.6	80 ^c	78	97.5	27	33.8
8	1.8	179	179	100	136	76.0
10	2.7	32	32	100	32	100
OD X CHI						
6	-	51 ^c	37	72.5	3	5.9
7	0.8	42	0		0	
9 ^d	2.6	54	3	5.6	3	5.6
10	2.6	11	0		0	
11	3.0	11	1	9.1	0	

a, b and c: as described in Table 26.

d: CHI-T58 was used as paternal parent.

***Z. aethiopica* (AEsp) X *Z. odorata* (OD)**

Only one inflorescence of AE_{sp} was pollinated with pollen of OD. The development of endosperms and embryos was observed at nine WAP. Embryos were about 2.7 mm long and endosperms were hardening. From 30 cultured embryos at nine WAP, 27 pale green plants had been produced. More than one hundred matured seeds were harvested and used in a seed germination test. The hybridity of five plants tested was confirmed by Southern hybridisation (Chapter 9).

***Z. odorata* (OD) X *Z. aethiopica* (AE)**

The endosperms developed normally in OD X AE. At seven WAP, the endosperms did not show much difference from those of OD X OD. The embryos, however, were much smaller than those of OD X OD, 0.7 mm long compared to 2.0 mm long. Most of the endosperms were normal until eleven WAP. Embryos were about 1.7 mm and 2.5 mm long at nine and eleven WAP respectively, smaller than embryos of OD X OD, 2.7 mm and 3.0 mm long at these times. A number of mature seeds were harvested. On average, 4.6 seeds per ovary were formed (Table 25).

Embryo germination on culture medium was very poor. Of 60 embryos cultured at seven WAP, only two produced pale green plants. Some embryos showed size increase with an abnormal shape at the beginning of culture, but did not generate any shoots. Of 62 embryos cultured at nine WAP, one generated white callus and others showed only a little enlargement. One plant generated from 68 embryos cultured at 11 WAP (Table 26)

***Z. odorata* (OD) X *Z. aethiopica* 'Childsiana' (CHI)**

The situation in OD X CHI was much the same as in OD X AE. The endosperms were normal and embryos were similar in size to OD embryos after ten WAP. Only a few embryos generated plants on the medium (Table 27). A number of seeds were harvested and used in a seed germination test (Section 3.3).

***Chromatella* (CHR) X *Z. odorata* (OD)**

The development of embryo and endosperm was much worse in CHR X OD than in AE X OD. The endosperms were starting to degenerate from five WAP. Up to seven WAP, many endosperms were watery and transparent. Many seeds were empty at ten WAP as a result of complete degeneration of endosperm. Before ten WAP, all embryos were found to be very small (less than 0.3 mm) (Table 28). After ten WAP, some ovules

were found to contain semi-soft endosperm and embryos with sizes from 0.5 -1.5 mm in length (Table 28).

Table 28. Development and *in vitro* culture of embryos of crosses between CHR or RE and OD ^a

WAP	embryo size(mm) <i>in vivo</i>	total embryo cultured	developed embryos ^b	generated plants
CHR X OD				
5	<0.3	30 ^c	1	0
6	<0.3	22 ^c	1	0
7	<0.3	153 ^c	23	0
8	<0.3	10 ^c	3	0
9	<0.3	80 ^c	15	2 albino
10	0.5-1.0	38	21	5 albino
12	0.5-1.0	22	8	2 albino
14	0.5-1.5	44	22	1 albino
RE X OD				
7	<0.3		7	1 albino
OD X CHR				
5	-	28 ^c	12	2 green ^d
6	0.9	24 ^c 24	19	0
7	1.5	19	7	3 green
9	2.4	21	5	4 green
10	2.8	129	0	0
*	2.8	57	57	57 green

a, b and c: as described in Table 26.

d: green plants resulted from self-pollinations.

*: Seeds were collected at 12 WAP and stored at room temperature for two months before the embryos were dissected and cultured.

Embryos embedded in the endosperms cultured before nine WAP did not form any calli or albino shoots (Table 28). However, approximately a half of the isolated embryos cultured at 10, 12 and 14 WAP formed calli or albino shoots. From some of

these embryos, the cotyledon expanded and formed callus but did not turn green. The callus did not regenerate. From other embryos, cotyledons expanded and remained white. White callus was generated from the haustorial tip of the cotyledon and albino leaves were generated from the embryo axis (Table 28).

***Z. rehmannii* (RE) X *Z. odorata* (OD)**

Only one inflorescence of RE was suitable for hybridisation when OD pollen was available. From this cross, development of embryos and endosperms was observed at seven WAP. Endosperms enlarged but were soft and watery. Embryos were very small (less than 0.3 mm). From 24 cultured embryos, seven showed a little increase in size and one generated an albino shoot (Table 28).

***Z. odorata* (OD) X *Chromatella* (CHR)**

The seeds of OD X CHR may be classified into two groups. One type of seed contained watery, degenerating endosperm or was empty because of the complete degeneration of the endosperm. A second type of seed contained normal endosperm. Embryos began elongating at five WAP. The embryos were about 0.9 mm, 1.5 mm, 2.4 mm and 2.8 mm long at six, seven, nine and ten WAP (Table 28). These embryos were smaller at six and seven WAP than the embryos of OD X OD although they reached normal embryo size after ten WAP. A number of mature seeds were harvested. On average, 1.7 seeds per ovary were produced (Table 25).

From five to nine WAP, 114 embryos were cultured on MS medium with 0.3 mg/l BA. Nine green plants were produced from these cultures (Table 28). At ten WAP, 129 well developed embryos (2.8 mm in length) were cultured but no plant was produced (Table 28). After mature seeds were stored in room temperature for two months, 57 embryos were dissected and cultured on the same medium. Most embryos were a good size and shape and 100% of embryos germinated and produced seedlings. The resulting plants were green (Table 28). Twenty-six green plants generated from OD X CHR were checked with an rDNA probe. All of them proved to be the product of self-pollination (Table 33). Thus the normal development of endosperm and embryo was the result of self-pollination, whereas strong incompatibility was present in hybrid endosperms and embryos. One embryo had a yellow cotyledon which later turned green. The first leaf was green but grew very slowly. The plant died soon after transferred to the greenhouse.

3.3. Seed germination

When fresh seeds from different crosses were tested for germination, good germination rates (more than 80%) were obtained from CHI X AE, AE X OD and AE_{sp} X OD within six weeks. Poor germination rates (less than 20%) were observed from OD X AE and OD X OD. The germination rate of OD X CHI (66.1%) was between the above two groups within six weeks. At 16 weeks, the germination rates increased to 32.5% from OD X AE and 66.9% from OD X OD (Table 29). After two months storage at room temperature, the germination rate of seeds from OD X OD was more than 90% at six weeks (Table 30).

Table 29. Germination of fresh seeds from different crosses

cross	No. of seeds	seeds germinated in 6 weeks		seeds germinated in 16 weeks	
		N.	%	N.	%
CHI X AE	91	86	94.5	-	-
AE X OD	238	192	80.7	-	-
AE _{sp} X OD	110	108	98.2	-	-
OD X CHI*	192	127	66.1	-	-
OD X AE	83	7	8.4	27	32.5
OD X OD	236	26	11.0	158	66.9

* A diploid-tetraploid chimera plant (CHI-T58) was used.

Seedlings of CHI X AE and OD X OD were green. Seedlings of AE X OD, AE_{sp} X OD and OD X AE were virescent. Virescent, albino and chimeric (virescent/albino) seedlings were produced from OD X CHI (Fig. 22). From 127 germinating seeds, 109 seedlings were established in a greenhouse. Twenty-five were virescent, 27 were albino and 57 were chimeric. All these seeds were originally from one inflorescence of OD pollinated with pollen from a diploid-tetraploid chimera plant of CHI (CHI-T58) (see Section 3.4 of Chapter 5). Diploid chromosome numbers were identified in two hybrids observed from this cross. It appeared that haploid pollen were applied in this cross. Virescent leaves of OD X CHI became green uniformly over the leaf tissue,

similar to the virescent leaves of CHI X OD. Virescent leaves of OD X AE became green initially on the leaf veins and subsequently over the rest of leaf tissue, similar to the virescent leaves of AE X OD.

Table 30. Germination of seeds of OD X OD and AE X CHI after two months storage

cross	GA ₃ (mg/l)	total seeds	seeds germinated	
			N.	%
OD x OD	0	40	36	90.0
OD X OD	6	40	36	90.0
OD X OD	25	40	39	97.5
OD X OD	100	40	40	100
AE X CHI	0	30	30	100



Fig. 22. Pale green, albino and chimeric hybrids of OD X CHI

CHI was a diploid-tetraploid chimera plant (CHI-T58).

4. Discussion

Three important reproductive characters of *Z. odorata* (OD) were observed in this study. Firstly, large sized tubers (approximately 3 cm in diameter) planted in the greenhouse did not flower without gibberellic acid (GA) treatment but produced one to three flowers per tuber when treated with 50 mg/l of GA₄₊₇ at the time of planting (Section 3.1). Perry (1989) reported that only one flower was produced from more than one hundred plants in cultivation over five years. The effects of GA on flowering of OD has not been reported previously. GA application was used to increase flower production from plants in section II (Corr & Widmer 1987, Funnell *et al.* 1988) by inducing flowers from axillary buds. But many flowers can be produced from the main buds and some axillary buds of big tubers even without GA treatment. From our observation, three genotypes (AE, AE_{sp} and CHI) flowered very well even without hormone application.

Secondly, the stigmas of OD were receptive when pollen is released from the same spadix. Emasculation was used in an attempt to prevent self-pollination in OD. It was impossible, however, to achieve complete emasculation in OD because female flowers are interspersed with numerous 'staminodes' which released pollen several days after the pollen released from the male zone (Section 3.1). To reduce the risk of self-pollination, it may be best to pollinate young flowers two or three times before pollen is released from the staminodes. By definition, staminode is a sterile stamen which does not produce pollen (Abercrombie *et al.* 1973). It is interesting to note that the staminodes described (Letty 1973, Perry 1989) in *Zantedeschia* do produce some pollen but the viability of this pollen has not been determined. It has been reported previously that the stigmas of *Z. aethiopica* lose pollen receptivity when pollen is released from the same spadix but the stigma of plants in section II can receive pollen from the same spadix (New 1964). Emasculation is much easier for plants in section II because female flowers are interspersed with only a few staminodes at the upper part of the female zone.

The third important reproductive character of OD was embryo dormancy. OD embryos (self-pollinated) germinated poorly and produced plants with weak growth when cultured at six and seven WAP and did not germinate when cultured at nine WAP (Table 24). Embryos dissected from OD seeds which were stored for two months gave 100% germination on the same medium. CHI embryos showed a 66% germination when cultured on the same medium at five WAP (Table 18) and a 100% germination

when cultured at nine WAP. These results indicate that the dormancy of OD embryos increases in the course of embryo maturation from six to nine WAP and breaks during the storage. At six or seven WAP, the dormancy is weaker hence a number of embryos generated plants albeit with weak growth. At nine WAP, strong dormancy blocks the germination of all embryos. In contrast, a lack of embryo dormancy in CHI resulted in an increase of embryo germination as they matured from five to nine WAP. Embryos of some plant species have a long dormant period after they mature. Excised dormant embryos have a low germination rate on nutrient medium and the resulting seedlings are abnormal in morphology and grow slowly (Hartmann & Kester 1983). The presence of embryo dormancy was supported by seed germination tests. Fresh OD seeds (self-pollinated) had a poor germination rate compared to fresh seeds of CHI X AE (Table 29). The germination rate (90%) of OD seeds was similar to that of seeds of CHI X AE after two-months storage at room temperature (Table 30). *Z. odorata* grows in an area of winter rainfall but dry summers (Perry 1989). The species flowers in late July so that seed matures just before the beginning of the dry season. The development of seed dormancy in the species may have evolved to prevent seed germination at this time.

Embryo dormancy was also present in OD X AE and OD X CHI. Embryos of these two crosses showed a poor germination (approximately 5%) when cultured at six and seven WAP and no germination when cultured at nine, ten or eleven WAP even though embryos were big (>2.0 mm long) (Tables 26 and 27). Fresh seeds of OD X AE and OD X CHI had a low seed germination rate than those of CHI X AE (Table 29). The reason for the poor germination should be embryo dormancy. In the reciprocal crosses CHI X OD and AE X OD, however, embryos germinated much better on the same medium (Tables 26 and 27). Fresh seeds of AE X OD and AE_{sp} X OD had a similar seed germination rate to CHI X AE (Table 29). Therefore there is no embryo dormancy in OD X section I. The genome ratio of two parents in the endosperm and plastome type in the hybrid change according to the cross direction. These changes may have effects on the differences in embryo dormancy in the reciprocal crosses. The seed germination rate of OD X CHI (66.1%) was much higher than that of OD X AE (8.4%). The genotype of the paternal parent may have an effect on hybrid embryo dormancy.

Crosses can be made in two directions between *Z. aethiopica* and OD. The development of embryos, however, was better with OD as the paternal parent than with OD as the maternal parent (Section 3.2). A large number of plants were generated with OD as the paternal parent whereas fewer plants were generated with

OD as the maternal parent (Tables 26, 27, and 29). Embryos of section I and section II can usually generate plants on MS medium with 0.3 mg/l BA when they are larger than 2.0 mm. Only a few embryos generated plants even though they were larger than 2.0 mm in crosses OD X AE and OD X CHI (Tables 26 and 27). This poor plant generation was possibly affected by embryo dormancy. Seed germination rate of OD X AE (32.5%) was much lower than that of OD X OD (66.9%) at 16 weeks in germination condition (Table 29). This lower germination rate may be affected by poor embryo development. Most of the hybrids produced between *Z. aethiopica* and OD were virescent. These virescent plants became green as they matured and produced flowers.

Two genotypes of *Z. aethiopica*, AE and CHI, revealed two types of virescence following crossing with OD. In the reciprocal crosses between OD and AE, the leaf veins became green sooner than the rest of leaf tissue (Fig. 21). In the reciprocal crosses between OD and CHI, greening occurred evenly over the leaf tissue. Virescence in AE_{sp} X OD was the same as that in CHI X OD. Albino and chimeric seedlings were produced from OD X CHI, but not from OD X AE. Only 32.5% seeds of OD X AE germinated and produced 27 plants (Table 29) after 16 weeks in germination. Albino embryos usually germinate more slowly than green embryos (New & Paris 1967). If a higher germination rate was achieved, albino plants may also be produced from OD X AE. If no albino plants are produced even though most seeds of OD X AE are germinated, then there must be differences between AE and CHI in plastome/genome incompatibility with OD. It was suggested that these albinos and chimeras were affected by incompatibility between the plastome and the genome. At least one difference between AE plastome and CHI plastome was detected on the basis of RFLP analysis (Table 32). Reciprocal crosses between AE and CHI, however, produced only green progeny (observation in this study). Plants generated from CHR X OD and RE X OD were albino. No hybrid was produced from OD X CHR. In the interspecific crosses within section II, most hybrids were green, but some hybrids were virescent, albino or chimera (New & Paris 1967).

From CHR X OD, endosperms were degenerating and watery. Embryos were generally small (< 0.3 mm) but a number of embryos were elongated to around 1.0 mm (Table 28). Embryos were rarely found reaching to 1.0 mm in crosses between section I and section II (Chapter 6 in this thesis, Chi 1990). Although an improvement in embryo development was obtained in CHR X OD compared to crosses between the two sections, hybrid albinism was not overcome in CHR X OD.

From OD X CHR, a large number of self-pollinations occurred even though the male zone was removed. Self-pollination, however, was not detected from OD X AE or OD X CHI, i.e. nine plants checked with rDNA probe were all hybrids (Chapter 9) and all progeny were virescent. The self-pollinations could be caused by later released pollen from the staminodes which could not be removed. Thus it appeared that all stigmas received pollen of AE or CHI and lost further receptivity when pollen was released from the staminodes, whereas some stigmas did not receive pollen of CHR and were receptive when pollen was released from the staminodes. Prefertilisation incompatibility could be a reason for the poor pollination with CHR pollen on OD stigma. But other possibilities for the self-pollination could not be ruled out.

Evidence obtained in this study supports the view that *Z. odorata* is an independent species. OD had a distinct karyotype from both *Z. aethiopica* (section I) and other species in section II (Chapter 4). The results of RFLP analysis with nuclear rDNA and ptDNA probes demonstrated that OD not only had DNA fragments common to each section but also had its own specific DNA fragments which were not present in either section (Table 32). Embryo dormancy was a distinct reproductive character of OD compared to all other species in *Zantedeschia*. Hybridisation incompatibility (shown as albino and virescent hybrids) was demonstrated between OD and three genotypes of *Z. aethiopica* but no incompatibility was observed among these genotypes of *Z. aethiopica*. This result indicates that OD is not a genotype of *Z. aethiopica* even though OD is most like *Z. aethiopica* morphologically. Strong incompatibility (only albino hybrids produced) was observed between OD and species in section II. All these results support the view that OD is not a variety of any species.

Crossing relationships in the genus *Zantedeschia* correspond with the phylogenetical relationships determined by other characters. The genus is divided into two distinct sections (Letty 1973) based on morphological characters and a new species, OD, falls between the two sections (Perry 1989). The two sections had distinct chromosome karyotypes even though karyotypes in section II were not distinct (Chapter 4). Based on karyotype asymmetry, OD was intermediate between section I and section II although it was more closely-related to section I than to section II. RFLP analysis using 18s rDNA as a probe revealed that species in section II had same bands and three genotypes in section I had same band (Table 32), thus clearly differentiated the two sections. Quite often more closely related species are more compatible (Bohs 1991). Postfertilisation barriers inhibiting embryo development and plastome/nuclear

incompatibility causing albinism were demonstrated in the crosses between the two sections (Chapter 6 in this thesis, Chi 1989). But the crosses between species within section II were compatible (Traub 1949, New & Paris 1967, Chi 1990). These hybridisation results indicate that species in section II are more closely related to each other than to *Z. aethiopica*. OD hybridised to section I and improved compatibility (based on embryo size, Section 3.2) to section II compared to crosses between section I and section II. This result indicates that OD is intermediate between the two sections but it is more closely related to section I than to section II. In the genus *Lupinus*, crossing relationships confirmed the chromosome number relationships between species (Roy & Gladstones 1988). The crossing relationships corresponded with morphological similarity between some species but not all species in the genus *Cyphomandra* (Bohs 1991).

This study has tested for the first time the possibility of using OD as a bridge for gene transfer between the two sections of *Zantedeschia*. A number of hybrids have been produced from reciprocal crosses between OD and *Z. aethiopica* but only albino hybrids have been produced from crosses between OD and CHR or RE. As OD falls between the two sections, hybrids could be more easily produced from crosses between OD and section II than from crosses between *Z. aethiopica* and section II. It may be worthwhile to try different genotypes from section II and/or pollen irradiation in order to overcome the incompatibility between OD and section II.

CHAPTER 8 Selection of nuclear and plastid DNA probes for hybrid characterisation

1. Introduction

It is necessary to determine whether the progeny resulted from wide crosses contain the genomes of both parents. Many methods, such as molecular biology methods, isozyme methods, cytogenetic methods and morphologic methods, have been used for this purpose (see Chapter 3 for details). Details on the methodology, application and advantages of molecular biology techniques in hybrid identification were reviewed in Chapter 3 and will not be repeated here.

Although maternal inheritance of plastid (pt) DNA is found in a majority of angiosperms, biparental inheritance of ptDNA occurs in about 20% of angiosperms (Corriveau & Coleman 1988, Harris & Ingram 1991). Paternal ptDNA inheritance has also been demonstrated in intraspecific crosses of *Medicago sativa* (Schumann & Hancock 1989, Masoud *et al.* 1990) and in interspecific crosses of the genus *Daucus* (Boblenz *et al.* 1990). Plastid DNA inheritance has not been studied previously in the genus *Zantedeschia*. One of the research interests of this study was to determine the ptDNA inheritance pattern in the *Zantedeschia* hybrids by RFLP analysis using cloned ptDNA as a probe. Deletions of ptDNA have been detected in albino plants regenerated from anther cultures of barley and wheat (Day and Ellis 1984, 1985), rice (Harada *et al.* 1991) and in albino plants regenerated from scutellar calli of *Hordeum marinum* (Shimron-Abarbanell & Breiman 1991). Another aim of this study was to determine whether ptDNA deletions occurred in albino, interspecific hybrids of *Zantedeschia*.

In this Chapter, the construction of a partial library using total leaf DNA of *Z. aethiopica* 'Childsiana' (CHI) is described. From this library, a species-specific nuclear DNA clone and a number of ptDNA clones were isolated. RFLPs (restriction fragment length polymorphisms) among the species of *Zantedeschia* were detected using labelled DNA from these ptDNA clones and an rDNA clone of apple. It was found that the species-specific clone and the rDNA clone were useful for identifying the hybrid nuclear genome and the ptDNA clones were useful for characterising the hybrid plastid genome (plastome).

2. Materials and methods

2.1. Extraction of total DNA from plant tissues

In most of the DNA isolation experiments in this study, young leaf tissue of plants growing in a greenhouse was used. In a few cases, green and albino leaf tissue of plantlets growing *in vitro* were used. In one case, roots of plants growing in a greenhouse were used. The amount of tissue varied from 30 milligrams to 15 grams. Plant DNA was extracted using a modification of the CTAB (cetyltrimethylammonium bromide) nucleic acid extraction method of Rogers and Bendich (1988).

CTAB extraction buffer and 10% CTAB solution were heated in a water bath at 65 °C. Leaf tissue weighing more than 0.2 gram was ground in dry ice or in liquid air with a mortar and pestle. Leaf tissue weighing less than 0.2 gram was ground in a plastic bag using a small wooden roller after the bag was chilled in liquid air. Ground tissue was transferred to a centrifuge tube and mixed with an equivalent volume (w/v) of CTAB extraction buffer (65 °C). For small samples, a 1.5 ml microfuge tube was used. The mixture was held at 65 °C for 5 minutes. One volume of chloroform/octanol (24:1) was then added. The tube was inverted several times to form an emulsion and then stored on ice for at least 15 minutes. The aqueous phase was separated from the chloroform by centrifuging the tubes for 30 seconds at 11000g. The aqueous phase at the top was transferred to a new centrifuge tube and the chloroform phase was discarded. (In some cases, a second chloroform extraction was performed by adding 1/10 volume of the 10% CTAB solution (65 °C) and one volume of chloroform/octanol followed by mixing and centrifugation). An equal volume of CTAB precipitation buffer was added to the aqueous phase and mixed gently. The tube was then placed on ice for at least 15 minutes. DNA was pelleted by centrifuging the tube at 15,000 rpm in a Sorvall SS-34 rotor, at 4°C for 15 minutes. The DNA pellet was dissolved in high-salt TE buffer and precipitated by adding two volumes of cold 95% alcohol. After centrifugation, the DNA pellet was resuspended in cold 70% alcohol (up to the original volume). The tube was placed in ice for 5-10 minutes and then centrifuged again. The supernatant was discarded and the DNA pellet was dried in a vacuum desiccator until all of the liquid had evaporated (about 10-30 minutes). The DNA was then dissolved in a small volume of 0.1X TE buffer. At this stage, the DNA sample was treated with RNase (Section 2.8.1), digested with restriction enzyme (Section 2.8.5), or stored at -20 °C.

Solutions

- CTAB extraction buffer: 2% CTAB (w/v), 100 mM Tris, 20 mM EDTA, 1.4 M NaCl, 1% PVP (polyvinylpyrrolidone) Mr 40000, pH 8.0
- 10% CTAB solution: 10% CTAB, 0.7 M NaCl
- CTAB precipitation buffer: 1% CTAB, 50 mM Tris, 10 mM EDTA, pH 8.0
- High-salt TE buffer: 10 mM Tris, 1 mM EDTA, 1 M NaCl, pH 8.0
- TE buffer: 1.0 mM Tris, 0.1 mM EDTA, pH 8.0

2.2. Construction of a partial genomic library

The library was constructed by ligating (Section 2.2.3) *Hind*III digested total leaf DNA of *Z. aethiopica* 'Childsiana' (CHI) (Section 2.2.2) into *Hind*III-digested, pGEM-3Z DNA (Section 2.2.1) and transforming (Section 2.2.5) the recombinant plasmids into competent *E. coli* DH5 α cells (Section 2.2.6). Colonies of transformed cells were selected on the basis of ampicillin resistance. Those containing recombinant plasmids were selected on the basis of alpha complementation of the β -galactosidase gene. The *lac* sequences present on pGEM-3Z code for a functional copy of the alpha peptide of β -galactosidase, which can combine with the host-encoded beta peptide to create functional β -galactosidase. In the case of non-recombinants, β -galactosidase utilizes the chromogenic substrate, X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactoside), resulting in blue colonies. When the coding region of the alpha peptide is insertionally inactivated by recombination of foreign DNA into the multi-cloning site located within the *lac* sequences, non-functional β -galactosidase is produced, resulting in white colonies. The selective medium also contains IPTG (isopropyl- β -D-thiogalactoside) which can induce the plasmid-borne *lac* promoter.

2.2.1. Preparation of cleaved pGEM-3Z vector DNA

An aliquot (2.5 μ g) of pGEM-3Z DNA was digested with *Hind*III (Section 2.8.5) in a total volume of 50 μ l. At 20 minutes before the restriction enzyme digestion was completed, 1 μ l (7.5 units) of CIP (alkaline phosphatase from calf intestine) was added. This dephosphorylation step was omitted for the first vector DNA preparation. After the digestion and the dephosphorylation were completed, 200 μ l of TE buffer was added to the reaction solution. The DNA was then purified and precipitated as described in Section 2.8.2 and 2.8.3. DNA was finally dissolved in 25 μ l of TE buffer. An aliquot (1

μl) of this digested DNA was checked on a 0.7% agarose gel to determine the degree of digestion and recovery of DNA.

2.2.2. Preparation of fragments for insertion

An aliquot containing 5 ug of total DNA of CHI was digested with *Hind*III in a total volume of 50 μl (Section 2.8.5). The DNA was then purified (Section 2.8.2), precipitated (Section 2.8.3) and finally dissolved in 25 μl of TE buffer. An aliquot (1 μl) of digested DNA was checked on a 0.7% agarose gel.

2.2.3. Ligation

Three types of ligation were performed as follows:

	ligation 1	ligation 2	ligation 3
<i>Hind</i> III cut pGEM-3Z DNA	5 μl	5 μl	5 μl
<i>Hind</i> III cut CHI DNA	10 μl	10 μl	0 μl
5X ligation buffer	10 μl	6 μl	6 μl
sterile distilled water	24 μl	8 μl	18 μl
ligase (1 unit/ul)	1 μl	1 μl	1 μl

The ligation mixture was incubated at 14-17 °C overnight. An aliquot (5 μl) of ligation mix was checked on a 0.7% agarose gel. In ligation 1, *Hind*III cut pGEM-3Z DNA was not treated with phosphatase. In ligation 2 *Hind*III cut pGEM-3Z DNA was treated with phosphatase. Ligation 3 was a control treatment.

Solution

- 5X ligation buffer: 330 mM Tris-HCl, 25 mM MgCl₂, 5 mM dithioerythritol, 5 mM ATP, pH 7.5.

2.2.4. Preparation of competent *E. coli* DH5 α cells

The procedure used was as follows:

1. Remove a single colony of *E. coli* DH5 α from the LB plate and inoculate into 5 ml LB liquid medium. Incubate the culture overnight at 37 °C with shaking.
2. Inoculate 40 ml LB liquid medium with 0.4 ml fresh overnight culture, and incubate with shaking at 37 °C until an A₅₅₀ of between 0.5 and 0.6 is reached (about 3 hours after incubation).
3. Centrifuge cells at 500 rpm in a Sorvall SS-34 rotor, at 4 °C for 10 minutes.
4. Resuspend the pellet in 20 ml, ice-cold, sterile, 50 mM CaCl₂ solution; allow to stand on ice for 30 min.
5. Centrifuge cells as described above (step 3).
6. Resuspend pellet in 4 ml ice cold sterile 50 mM CaCl₂ solution. These competent cells can be stored on ice for up to 24 hours, or used for transformation immediately.

Media

LB (Luria-Bertani) Medium: Dissolve 10 g Bacto tryptone (Difco), 5 g Bacto yeast extract and 5 g NaCl in distilled water and adjust volume to 1 liter. Autoclave at 1.1 kg/cm² for 15 minutes.

LB plate: add 15 g Bacto agar (Difco) to 1 liter LB liquid medium, autoclave, cool to about 50 C, pour 25 ml into sterile petri dishes and allow to solidify.

2.2.5. Transformation of DH5 α cells

The procedure used was as follows:

1. Mix the 10 μ l of ligated DNA (Section 2.2.3) with 40 μ l of Tris-HCl (50 mM, pH 7.2) in an ice-cold microfuge tube.
2. Use 10 ng circular pGEM-3Z DNA in 50 μ l Tris-HCl for a positive control and no DNA (only 50 μ l Tris-HCl) for a negative control.
3. Add 300 μ l competent *E. coli* DH5 α cells, mix gently, and incubate on ice for 40 minutes.
4. Heat the tube in a 42 °C water bath for 3 minutes and then let it stand at room temperature for 10 minutes.
5. Add 650 μ l LB liquid medium and shake for 60 minutes at 37 °C.
6. Distribute an aliquot, typically 100-200 μ l, of cells on LB/Amp/X-gal/IPTG plates.

7. Incubate plates upside down at 37 °C overnight. Typical transformation efficiency was approximately 1000 colonies per 1 ng pGEM-3Z DNA (in the positive control).

Media

LB/Amp plate: Add 1/100 volume of 10 mg/ml ampicillin solution (sterile filtered) to the LB medium with agar (Section 2.2.4) before pouring the medium into plates.

LB/Amp/X-gal/IPTG plate: To a cold LB/Amp plate, add 40 µl of X-gal (20 mg/ml dissolved in dimethylformamide) and 40 µl of IPTG (24 mg/ml dissolved in water, sterile filtered) and immediately spread the solution over the plate surface with a glass rod.

2.2.6. Preservation of bacterial cultures

Short time storage: Colonies on LB or LB/Amp plates were stored at 4 °C for from several weeks to a few months.

Long term storage: An aliquot containing 0.85 ml of overnight culture was thoroughly mixed with 0.15 ml of sterile glycerol in a sterile microfuge tube and stored at -70 °C.

2.2.7. Storage of colonies on a membranes

The procedure for storage of colonies on a membrane was similar to that described by Hanahan and Meselson (1983). Template membrane (see Section 2.6.1.1) was transferred to an F plate (LB plate with the addition of 5% glycerol) and incubated for a few hours at 25 °C. Sterile, dry, nylon membranes were pre-wetted on an F plate just prior to use. A replicate membrane was made by laying a pre-wetted membrane onto the template membrane and pressing gently. The two membranes were left together and sandwiched between several dry Whatman filters, plus one wet whatman filter to maintain humidity. The stack was placed inside a plastic bag, sealed and stored at -70 °C.

2.3. Extraction of bacterial plasmid DNA

2.3.1. Minipreparation of bacterial plasmid DNA

Small quantities of plasmid DNA were isolated according to the rapid boiling method of Holmes and Quigley (1981). A single colony of transformed *E. coli* cells was inoculated into LB/Amp liquid medium (typically 4 ml) at 37 °C overnight. Cells were pelleted from 1.5 ml of the overnight culture by centrifugation in a microfuge tube for one minute. The supernatant was poured off and the tube was drained against paper towels. The cell pellet was resuspended in 350 µl of STET buffer and 25 µl of lysozyme solution was added and mixed. The cell mix was boiled for 40 seconds and then centrifuged immediately for 10 minutes in a microfuge tube. The gelatinous pellet was removed with a toothpick and an equal volume of cold isopropanol (usually about 300 µl) was added. The tube was inverted several times and placed at -20 °C for 10-20 minutes. The DNA was pelleted in a microfuge for 5 minutes and washed with 350 µl of cold 70% alcohol. The DNA was then dissolved in TE and treated with RNase (Section 2.8.1).

Medium

- LB/Amp liquid medium: Cool the LB medium (Section 2.2.4) to about 50 °C after autoclaving and add 1/100 volume of 10 mg/ml ampicillin solution (sterile filtered).

Solutions

- STET buffer: 8% sucrose, 5% Triton X-100, 50 mM EDTA, 50 mM Tris-HCL, pH 8.
- Lysozyme solution: 10 mg/ml freshly prepared in 10 mM Tris, pH 8, store on ice.

2.3.2. Large scale preparation of plasmid DNA

The large scale preparation of plasmid DNA was based on the method described in 'Protocols and Applications 1989/1990', Promega. A single colony of pGEM-3Z transformed *E. coli* DH5α was picked from a plate, inoculated into 50 ml of LB/Amp liquid medium and cultured overnight in a shaker at 37 °C. Cells were harvested by centrifugation at 5000 rpm in a Sorvall SS-34 rotor for 15 minutes at 4 °C. The cells were thoroughly resuspended in 1.5 ml of ice cold, freshly prepared lysis buffer by pipetting them up and down with a pipette and were then incubated on ice for 10 minutes. To the cells, 3 ml of freshly prepared 0.2 N NaOH and 1% SDS was added and gently mixed by inversion and then the mix was incubated on ice for 10 minutes.

Sodium acetate (1.9 ml), 3 M, pH 4.6, was added and mixed by inversion and placed on ice for 20 minutes. The mix was centrifuged at 12,000 g for 15 minutes and the supernatant was transferred to a fresh tube. RNase A (12 μ l of a 1 mg/ml stock solution) was added to the supernatant and the mix was incubated for 20 minutes at 37 °C. The DNA was purified with phenol/chloroform (Section 2.8.2) and precipitated with ethanol (Section 2.8.3). The DNA pellet was re-dissolved in 400 μ l of H₂O and precipitated by mixing with 100 μ l of 4 M NaCl and 500 μ l of 13% polyethylene glycol (MW 8,000). After a one hour incubation on ice, DNA was harvested by centrifugation at 15,000 rpm in a Sorvall SS-34 rotor, at 4 °C for 15 minutes. The DNA was then washed with 1 ml of 70% alcohol, dissolved in an appropriate volume of TE buffer and stored at -20 °C.

Solution

- Lysis buffer: 25 mM Tris-HCL, 10 mM EDTA, 15% sucrose, 2 mg/ml lysozyme, pH 8.0

2.3.3. Preparation of insert DNA in agarose gels

To make a probe from either the insert DNA or from a particular part of the insert DNA, the DNA fragment of insert was isolated after agarose gel electrophoresis. The method used was based on the method described in "DNA Labelling and Detection---Nonradioactive" (Application Manual, Boehringer Mannheim).

The recombinant plasmid DNA was digested with the appropriate restriction enzyme(s) (Section 2.8.5) and the resulting DNA fragments were separated on an agarose gel (Section 2.8.6) using low gelling temperature agarose (Sea Plaque, FMC). An agarose block containing the DNA fragment of interest was cut out with a scalpel. The agarose block was incubated at 70 °C in a water bath for 10 minutes to totally melt the agarose. The melted agarose was extracted twice with the same volume of pre-warmed (37 °C), equilibrated phenol (Section 2.8.2) and once with chloroform/octanol alcohol (24:1). After each extraction, centrifugation was performed to separate precipitated agarose particles from the aqueous phase. The DNA in the top aqueous phase was precipitated by mixing it with 1/10 volume of 4 M LiCl and 2 volumes of 95% ethanol. The DNA was pelleted by centrifugation at 15,000 rpm in a Sorvall SS-34 rotor, at 4°C for 15 minutes, washed once with 70% alcohol, dissolved in an appropriate volume of TE buffer and stored at -20 °C.

2.4. Restriction enzyme mapping

A number of restriction enzymes which either do not cut pGEM-3Z or cut only within the polylinker region were chosen to digest the insert DNA of recombinant plasmids. Enzymes which cut the insert DNA at 1 to 3 locations were selected for mapping. Recombinant plasmid DNA was first digested with *Hind*III to release the insert DNA. The insert DNA was then digested further with a single restriction enzyme or with a combination of two restriction enzymes (Section 2.8.5). The digested DNA fragments were separated on an agarose gel (Section 2.8.6). From the photograph of the gel, the mobility of each DNA fragments was measured. The mobility of DNA markers was plotted against their size on a semi-logarithmic graph paper. The size of each fragment was determined by comparing its mobility with those of the markers. According to the size of the fragments, the restriction sites were mapped using a method similar to that described by Perbal (1988).

2.5. DNA labelling

2.5.1. Random primers α -³²P DNA labelling

A random primers DNA labelling system (BRL Life Technologies, Inc.) was used in this study following the manufacturer's recommendations:

1. Add 1 μ l (25-50 ng) DNA and 22 μ l H₂O to a microfuge tube. Denature the DNA by boiling for 7 minutes, then immediately cool on ice.
2. Perform the following addition on ice:
 - 2 μ l dATP solution
 - 2 μ l dGTP solution
 - 2 μ l dTTP solution
 - 15 μ l random primers buffer mixture
 - 5 μ l (approximately 50 uCi) [α -³²P]dCTPMix briefly.
3. Add 1 μ l Klenow Fragment, mix gently but thoroughly, and centrifuge briefly.
4. Incubate at 37 °C for at least one hour and then add 5 μ l of stop buffer.
5. Determine the incorporation of radioactivity using PEI-Cellulose Thin Layer Chromatography (see below).
6. Denature the labelled DNA by heating at 75 °C in the presence of 50% formamide, cool on ice and then use for hybridisation (Section 2.6.1.2).

Components of the system

- Random primers buffer mixture: 0.67 M HEPES, 0.17 M Tris-HCl, 17 mM MgCl₂, 33 mM 2-mercaptoethanol, 1.33 mg/ml BSA, 18 A₂₆₀ units/ml (1 A₂₆₀ unit/ml approximately equals 50 ug/ml) oligodeoxyribonucleotide primers (hexamer fraction), pH 6.8.
- dATP solution: 0.5 mM dATP in 3 mM Tris-HCl, 0.2 mM Na₂EDTA, pH 7.0
- dCTP solution: 0.5 mM dCTP in 3 mM Tris-HCl, 0.2 mM Na₂EDTA, pH 7.0
- dGTP solution: 0.5 mM dGTP in 3 mM Tris-HCl, 0.2 mM Na₂EDTA, pH 7.0
- dTTP solution: 0.5 mM dTTP in 3 mM Tris-HCl, 0.2 mM Na₂EDTA, pH 7.0
- Klenow Fragment (large fragment of DNA polymerase I): 3 units/ul Klenow Fragment in 100 mM potassium phosphate buffer, 10 mM 2-mercaptoethanol, 50% (v/v) glycerol, pH 7.0
- Stop buffer: 0.2 M Na₂EDTA, pH 7.5

PEI-cellulose thin layer chromatography

After a one hour incubation, a sample (approximate 0.1 µl) was removed and spotted onto an origin mark on a 4 X 8 cm (approximately) sheet of PEI-cellulose (polyethyleneimine-cellulose coated plastic sheets for thin layer chromatography; Merck #5579). The samples were air dried, chromatographed for 10-15 minutes in a covered beaker containing approximately 2 mm (depth) of 0.75M KH₂PO₄ adjusted to pH 3.5 with orthophosphoric acid. The PEI chromatogram was wrapped in plastic film and autoradiographed with X-ray film (RX, Fuji) for 30 minutes. The X-ray films were developed as described in Section 2.8.7. This method was modified from Reed's procedures (K.C. Reed, Australian National University, 1988, unpublished data).

2.5.2. Chemiluminescence DNA labelling

An ECL (enhanced chemiluminescence) Gene Detection System (Amersham) was used for DNA labelling. The procedure used followed the manufacturer's recommendations:

1. Using the water supplied, dilute the DNA to be labelled to 10 ng/ul. The amount of DNA to be labelled is dependent on the size of the membrane to be probed (normally 0.25-0.5 ug DNA for a 100 cm² membrane).

2. Denature the DNA by boiling for 7 minutes, then immediately cool the DNA on ice for 5 minutes. Spin briefly (5 seconds) in a microfuge.
3. Add an equivalent volume of labelling reagent (containing horseradish peroxidase) to the cold DNA. Mix thoroughly.
4. Add the glutaraldehyde solution, using a volume equivalent to the volume of labelling reagent. Mix thoroughly and spin briefly.
5. Incubate for 10 minutes at 37 °C. If not used immediately, this labelled DNA can be held on ice for a short period (for example 10-15 minutes).

2.6. Membrane-based DNA/DNA hybridisation

2.6.1. Colony hybridisation

2.6.1.1. Making template and replica membranes

The procedure used was as follows:

1. Cut nylon membrane (Hybond-N⁺, Amersham) to fit the plate and autoclave.
2. Place a sterile membrane onto an LB/Amp plate (Section 2.3.1).
3. Using sterile toothpicks, transfer the white colonies from LB/Amp/X-gal/IPTG plates (Section 2.2.5) onto the membrane.
4. Incubate the plate upside down at 25 °C overnight to achieve small colonies (approximately 0.1 mm in diameter).
5. Place a fresh membrane onto the older membrane, press gently and peel off. Store the old membrane as a master membrane. Incubate the new membrane overnight at 25 °C and then use it for colony hybridisation.

2.6.1.2. Binding liberated DNA to the membrane

The colony hybridisation membrane (Section 2.6.1.1) was laid, colony face up, on Whatman No 1 paper soaked in 2X SSC/0.1% SDS in a petri dish for 2 minutes. The dish was transferred to a microwave oven and treated for 2.5 minutes at full setting (650 watts) thereby lysing cells and simultaneously denaturing and fixing the DNA to the membrane (Buluwela *et al* 1989). The membrane was then wetted with 5X SSC/0.1% SDS and soaked in the pre-washing solution for 1.5 hours at 42 °C with shaking to remove the cell debris (Maniatis *et al*. 1982).

Solutions

- Pre-washing solution: 50 mM Tris-HCl, 1 M NaCl, 1 mM EDTA, 0.1% SDS, pH 8.0.
- 20X SSC: 0.3 M Na₃Citrate, 3 M NaCl, pH 7.0.

2.6.1.3. Hybridisation

The pre-washed membrane (Section 2.6.1.2) was placed in a plastic hybridisation bag. The membrane was pre-hybridised with hybridisation buffer (using 10 ml per 100 cm² of member) at 42 °C with shaking. After 1-2 hours incubation, radioactively labelled probe (Section 2.5.1) was added and the incubation was continued overnight at 42 °C with shaking. After the hybridisation buffer was removed, the membrane was washed twice with 2X SSC/0.2% SDS for 20 minutes at room temperature and then washed twice with 2X SSC/0.2% SDS for 30 minutes at 65 °C. The membrane was then placed on paper towels to remove excess solution and transferred to a piece of 3MM paper and wrapped with polyethylene film. The hybridisation signal, measured on a mini-monitor, determined the period required for exposure of the membrane to an X-ray film (RX Fuji). The autoradiography was carried out at -70 °C for 2-7 day. The X-ray film was developed as described in Section 2.8.7.

Solutions

- Denhardt's Solution (50X): Dissolve 5 g ficoll, 5 g polyvinylpyrrolidone (pvp) and 5 g BSA (Pentax Fraction V) in 100 ml distilled water.
- Herring sperm DNA: Dissolve the DNA (herring sperm DNA, Boehringer) in water at a concentration of 10 mg/ml. Shear the DNA by passing it several times through an 18-gauge hypodermic needle. Boil the DNA for 10 minutes and store at -20°C in small aliquots. Just before use, heat the DNA for 5 minutes in a boiling-water bath and then chill quickly in ice water.
- Hybridisation buffer: 50% formamide, 5X Denhardt's solution, 5X SSC, 0.1% SDS, 400 ug/ml herring sperm DNA. Make freshly using distilled water and use the buffer at the rate of 10 ml per 100 cm² of membrane.

Note: If the membrane was to be reprobed, it was never allowed to dry out. Before reprobing, the membrane (Hybond-N⁺) was incubated in 0.4 M NaOH at 45 °C for 30 minutes, transferred to 0.1X SSC, 0.1%(w/v) SDS, 0.2 M Tris-HCl, pH 7.5 and incubated for a further 15 minutes at 45 °C. (This method was based on the method described in "Blotting and hybridisation protocols for Hybond membranes", Amersham)

2.6.2. Dot hybridisation

2.6.2.1. Preparation of dot blots

Dot blots were made with a Bio-Dot apparatus (Bio-Rad). The procedure used followed the manufacturer's recommendations in the manual for the apparatus.

1. Denature DNA by boiling for 5 minutes and then chill in ice water.
2. Add one volume of 2 M ammonium acetate to the denatured DNA. Make a dilution series of the DNA with 1 M ammonium acetate.
3. Assemble membrane apparatus (as described in the manual) with either a nitrocellulose membrane (BAS85, Schleicher & Schuell) or nylon membrane (Hybond-N⁺, Amersham), pre-wetted and equilibrated in 1M ammonium acetate.
4. Apply serial diluted DNA into appropriate wells with vacuum off but flow valve open. Normally 200 µl of DNA solution containing not more than 0.5 ug DNA is applied into each well. Cover unused wells with parafilm.
5. Allow the DNA to bind to the membrane by applying a gentle vacuum.
6. Add 200 µl of 1 M ammonium acetate into each well, apply vacuum and maintain vacuum until the wells are dry.
7. Remove the membrane from the apparatus. Fix the DNA to the membrane as described in step 12 for Southern blotting (Section 2.6.3.1).

2.6.2.2. Hybridisation and signal detection

An ECL (enhanced chemiluminescence) Gene Detection System (Amersham) was used for dot blot hybridisation. The procedure used followed the manufacturer's recommendations in the manual for the system.

1. Take the hybridisation buffer that is supplied and add NaCl to a final concentration of either 0.3 M or 0.5 M. For Hybond-N⁺ membrane, also add the block reagent to 5% w/v.
2. Place the membrane (dot blot in Section 2.6.2.1) in a plastic bag and add the above hybridisation buffer at a rate of 1.25 ml per cm² of membrane. Remove as many bubbles as possible and seal the bag.
3. Incubate the bag with shaking at 42 °C for 15 minutes for a nitrocellulose membrane and 1 -2 hours for a Hybond-N⁺ membrane.

4. Cut a corner of the bag, add chemiluminescence-labelled probe (Section 2.5.2) at a rate of 0.25 ug probe DNA per 10 ml of hybridisation buffer. Remove bubbles and seal the bag.
5. Incubate the bag at 42 °C overnight with shaking.
6. Remove the blot from the hybridisation medium, place in a clean container and cover with an excess of primary wash buffer (6 M urea, 0.5X SSC, 0.4% SDS). Incubate with agitation at 42 °C for 20 minutes.
7. Discard the wash buffer, replace with an equivalent volume of fresh wash buffer and incubate with agitation for a further 20 minutes at 42 C.
8. Discard the wash buffer. Place the blot in a fresh container, rinse with 2X SSC and incubate with agitation for 5 minutes at room temperature.
10. Discard the 2X SSC and replace with fresh 2X SSC. Incubate for a further 5 minutes at room temperature.
11. In a dark room, drain the 2X SSC from the blot and place the blot in a fresh container, DNA side up. Add the detection reagents directly to the membrane. Make the detection reagents by mixing equal volumes of detection solution 1 with detection solution 2 to give sufficient to cover the membrane (typically 0.0625-0.125 ml per 1 cm² of membrane).
12. Incubate for precisely 1 minute at room temperature.
13. Drain off excess detection solution and wrap the membrane in plastic sheet. Gently smooth out air pockets.
14. Place the blot, DNA side up, in a film cassette. Turn off the light and place a sheet of X-ray film (RX, Fuji) on the top of the membrane, close the cassette and expose for at least 1 minute. Replace with a fresh film and expose for a suitable time for optimal signal intensity. Sometimes, a third or fourth film may be necessary.
15. Develop the films as described in Section 2.8.7.

Note: Membrane can be reprobed without removing the previous probe.

2.6.3. Southern hybridisation

2.6.3.1. Preparation of Southern blots

1. Digest DNA with restriction enzymes (Section 2.8.5) and separate the resulting DNA fragments on an agarose gel (Section 2.8.6).
1. Following electrophoresis, photograph the gel to record the electrophoretic separation of the loaded DNA samples. Then, slide the gel from the gel-former into a suitable plastic box.
2. Cover the gel with denaturation solution (1.5 M NaCl, 0.5 M NaOH). Gently agitate for 1 hour.
3. Discard the denaturation solution and rinse the gel 3 times with distilled water.
4. Cover the gel with neutralization solution (1.5 M NaCl, 0.5 M Tris-HCL, pH 7.5) and gently agitate for 1 hour.
5. Discard the neutralization solution and rinse the gel 3 times with distilled water.
6. Fill a plastic box with an appropriate volume of 20X SSC and place a glass plate across the box to support the gel. Make blotting wicks with 3MM paper. The blotting wicks are 3mm wider than the width of the gel. Wet the wicks one by one in 20X SSC and lay 3 wicks across the glass plate on top of one another. Make sure both ends touch the 20X SSC solution in the box.
7. Pour sufficient 20X SSC over the top wick to flood it, then transfer the gel onto the wicks. Exclude all bubbles from between the wick and the gel and then surround the gel with polyethylene film.
8. Pour sufficient 20X SSC over the gel to flood it. Cut the membrane (BAS85 or Hybond-N⁺) to the same size of the gel. When using BAS85 membrane, wet it in distilled water first and then equilibrate in 20X SSC. Use dry Hybond-N⁺ membrane. Place the membrane over the gel. Exclude all bubbles.
9. One by one, wet 3 sheets of 3MM paper, cut to the same size of the gel, with 20X SSC and lay them on the top of the membrane. Again exclude all bubbles.
10. Lay a wad of paper towels on the 3MM paper and place a weight of approximately 200 g on top of the paper towels. Leave to blot overnight.
11. Dismantle blotting stack down to membrane. Mark the membrane so that the face carrying the DNA is distinguishable. Peel off the membrane.
- 12.a. Place nitrocellulose membrane in 6X SSC and wash for 5 minutes with gentle agitation. Lay the membrane, DNA side up, on a clean piece of 3MM paper and allow surface liquid to drain, then remove to a second piece of 3MM paper and

allow to air dry. Sandwich the blot between fresh 3MM paper and bake dry (80 °C for 2 hours) in a vacuum oven.

- 12.b. Place Hybond-N⁺ membrane on a pad of 3MM paper 2-3 piece thick soaked in 0.4 M NaOH for 2 minutes. Rinse the membrane briefly by immersion in 5X SSC with gentle agitation (maximum time 1 minute). Then place the membrane on a piece of 3MM paper to air dry.

2.6.3.2. Hybridisation and signal detection

The procedures used were the same as those described in either Section 2.6.2.2 or Section 2.6.1.3.

2.7. Isolation and characterisation of ptDNA clones

2.7.1. Selection of putative ptDNA clones

One leaf cell contains many chloroplasts and each chloroplast contains multiple-copies of ptDNA. The copy number of ptDNA in a green leaf cell of rice or a green suspension culture cell of tobacco is over 10,000 (Day & Ellis 1984, Cannon *et al.* 1985). When labelled total leaf DNA is used to probe a library derived from total leaf DNA, clones containing ptDNA and repetitive nuclear DNA show strong signals in colony hybridisation whereas clones containing single copy or low copy number of nuclear DNA sequences show weak signals.

The CHI library (see Section 2.2 for construction) contained ptDNA clones since total leaf DNA was used for this library construction. After the colony hybridisation using labelled total leaf DNA as a probe (Section 2.6.1), a number of clones showed strong hybridisation signals. From these clones, plasmid DNA was prepared by the minipreparation method (Section 2.3.1). Plasmid DNA was treated briefly with DNase I to nick the circular DNA molecules and then denatured in boiling water for 5 minutes. The DNase I treatment was included to produce single stranded linear DNA after the denaturation. Denatured recombinant plasmid DNA, as well as pGEM-3Z DNA (used as a control) were dot blotted onto nitrocellulose membrane (BAS85) (Section 2.6.2.1). The dot blot was probed with chemiluminescence-labelled (Sections 2.5.2 and 2.6.2.2) DNA of 8 kiwifruit ptDNA clones (Section 2.9.1). A DNA sample that showed a good signal with this probe was selected as a putative ptDNA clone.

2.7.2. Confirmation of ptDNA clones

A Southern blot was made (Section 2.6.3.1) with restriction enzyme digested DNA of albino hybrid No43 (embryo axis origin) and No47 (haustorium origin) of *Z. 'Chromatella'* (CHR) X CHI (Chi 1990). Shoots of No43 had ptDNA inherited from the two parents and shoots of No47 had ptDNA inherited from only the maternal parent although shoots from both sources had nuclear DNA inherited from the two parents (Chapter 9). Each putative ptDNA clone selected in Section 2.7.1, including pZAC1, pZAC2 and 4 other clones, was chemiluminescence-labelled (Section 2.5.2) and the resulting probe was hybridised (Section 2.6.2) to the Southern blot. Each probe revealed different bands between the two parents in the Southern hybridisation. When a probe revealed DNA bands inherited from both parents in hybrid No. 43 but only from the maternal parent in hybrid No47, that probe was considered to contain cpDNA. When a probe revealed DNA bands inherited from the two parents in both hybrid No43 and hybrid No47, that probe was considered to contain nuclear DNA.

2.7.3. Determination of the physical position of ptDNA clones on the plastome

A dot blot was made with DNA from the 8 kiwifruit ptDNA clones, leaf DNA and root DNA of CHI, and lambda DNA as a control. The blot was probed and reprobed (Section 2.6.2.2) with chemiluminescence-labelled (Section 2.5.2) DNA of pZAC1 and pZAC2. The kiwifruit ptDNA probes which hybridised to pZAC1 or pZAC2 were identified. The location of the kiwifruit ptDNA clones in the plastome (plastid genome) have been determined (Crowhurst *et al.* 1990). Most higher plants are similar in plastome structure (Palmer 1990). Therefore, the location of pZAC1 and pZAC2 in the plastome was determined accordingly.

2.8. Commonly used molecular biological techniques in this study

2.8.1. RNase treatment of DNA samples

A DNA sample containing RNA was treated with 1/10 volume of 1 mg/ml RNase A for at least 1 hour at 37 °C. After the treatment, the DNA was further purified by phenol/chloroform extraction (Section 2.8.2) and then ethanol precipitated (Section 2.8.3).

Solution

- RNase A: Dissolve pancreatic RNase A at a concentration of 1 mg/ml in 10 mM Tris-HCl, 15 mM NaCl, pH 7.5. Heat at 100 °C for 15 minutes. Allow to cool slowly to room temperature. Dispense into aliquots and store at -20 °C.

2.8.2. DNA purification by phenol/chloroform extraction

To a tube containing the DNA solution, 1/2 volume of phenol and 1/2 of chloroform/octanol (24:1) were added. The tube was inverted several times to form an emulsion and centrifuged to separate the phases. The top aqueous phase containing the DNA was transferred to a fresh tube containing one volume of chloroform/octanol. The tube was inverted and centrifuged again. The DNA in the top phase was transferred to a fresh tube and precipitated with ethanol (Section 2.8.3).

Solution

- Phenol: equilibrate phenol in 0.1 M Tris-HCl, pH 8.0 using the method described by Sambrook *et al.* (1989).

2.8.3. Precipitation of DNA with ethanol

To a tube containing the DNA solution, 2 volumes of alcohol (95%) and 1/10 volume of 3 M Sodium acetate (pH 5.2), or 4 M of LiCl were added and mixed. The tube was placed at -20 °C for at least 1 hour. DNA was pelleted by centrifugation and resuspended in 2 volumes of 70% ethanol. The tube was placed on ice for at least 10 minutes and then spun. The DNA pellet was dried under vacuum, dissolved in TE and stored at -20 °C.

2.8.4. Determination of DNA concentration

Two methods were used to determine DNA concentration. The first method employed spectrophotometry. An aliquot (1 - 5 µl) of a DNA sample was diluted to 1 ml with TE buffer. The value of A_{260} and A_{280} was determined. DNA concentrations were determined on the basis that 1 A_{260} unit corresponds to approximately 50 µg/ml of dsDNA in a 1 cm path length. The ratio of A_{260}/A_{280} was used as an indicator for the purity of the DNA samples (see Section 3.1). In the second method, the DNA sample was compared with a known amount of a DNA standard on an agarose gel (Section 2.8.6). A dilution series of both the DNA sample and the DNA standard was

applied on the same gel. The gel was run for a short time to move the DNA just into the gel. The DNA concentration was determined by comparing the fluorescence intensity of DNA samples to those of the DNA standard after a photograph was taken.

2.8.5. Restriction enzyme digestion

A DNA sample was digested with restriction enzyme, using either the buffer supplied by the manufacturer with each enzyme or an alternative buffer (L, M, or H) (Maniatis *et al.* 1982) if no buffer was supplied. If the DNA was to be digested with more than one enzyme, the digest requiring least salt was performed first and then additional salt (1 M NaCl) and the second enzyme were added. For multiple digests where this was not possible, the DNA was purified by ethanol precipitation (Section 2.8.3) after the first digest and then digested with the second enzyme. Normally 2 units of enzyme were used per microgram of DNA. Digestion was carried out for 2-5 hours at 37 °C. The reaction was stopped either by adding sample loading buffer (Section 2.8.6) when the DNA was to be used for gel electrophoresis, or phenol/chloroform extraction (Section 2.8.2) when the DNA was to be used for ligation.

Buffers

- 10X L buffer: 100 mM Tris-HCl, 100 mM MgCl₂, pH 7.5
- 10X M buffer: 100 mM Tris-HCl, 100 mM MgCl₂, 500 mM NaCl, pH 7.5
- 10X H buffer: 100 mM Tris-HCl, 100 mM MgCl₂, 1 M NaCl, pH 7.5

2.8.6. Agarose gel electrophoresis

Agarose (Ultra Pure, BRL) (typically 0.8%) was dissolved in TAE buffer in a microwave oven. When the agarose solution cooled to about 65 °C, 1 µl of ethidium bromide solution (0.5 mg/ml) was added per 1 ml of gel solution. The gel was then poured and allowed to solidify. The gel running buffer consisted of TAE buffer containing 0.5 µg/ml ethidium bromide. Each DNA sample was mixed with 1/10 volume of 10X loading dye in a microfuge tube, loaded into a well of the gel and electrophoresed (typical 3 volts/cm for running the gel overnight or 8 volts/cm for running the gel in day time). Following the electrophoresis, the gel was viewed on a UV transilluminator (Ultra-violet Products Inc.) and photographed with a Polaroid MP4 camera using Polaroid 667 film.

Solutions

- Tris-acetate electrophoresis buffer (TAE): 40 mM Tris-HCl, 20 mM acetate acid, 2 mM EDTA, pH 8.1
- 10X gel loading dye: 50% glycerol, 125 mM EDTA, 0.1% SDS, 0.05% (w/v) bromophenol blue

2.8.7. Development of X-ray film

In a dark room, the X-ray film (RX, Fuji) was removed from a film cassette under red safety light. The film was developed for 5 minutes in a developer (G150, Agfa), rinsed in 1% acetic acid solution and fixed for at least 10 minutes in an X-ray fixer (XF-25, Olin Hunt Specialty Products Pty Ltd). The film was then extensively washed in running water and air dried.

2.9. DNA clones obtained from other laboratories

2.9.1. Kiwifruit ptDNA clones.

Eight ptDNA clones of kiwifruit (*Actinidia deliciosa*) were kindly supplied by Dr. R. Gardner (Auckland University, New Zealand). These clones were labelled as K4, K6, K15, K17, K19, K24, K41, and K42 (K4 = λ kiwi4, K6 = λ kiwi6, etc.). The inserts of these clones combined represent about 70% of the kiwifruit plastome. The position of each insert on the plastome of kiwifruit is indicated in Fig. 38.

2.9.2. Apple 18s rRNA gene.

DNA of a 1.5 kb EcoR-1/XbaI fragment of the rRNA gene of apple (see Simon & Weeden 1992) was kindly provided by Dr S. Gardiner (HortResearch, Palmerston North, New Zealand).

3. Results

3.1. DNA isolation

Using the method in Section 2.1, total cellular DNA samples were prepared from *Zantedeschia* species and hybrids. These DNA samples contained predominantly nuclear DNA, as well as some plastid DNA (ptDNA) and mitochondrial DNA. In the following Sections, these DNA samples will be simply referred to as total DNA.

The DNA concentration was determined by spectrophotometry or comparison with a DNA standard (Section 3.1.4). Based on the DNA concentration, DNA yield was calculated. Typically 30 ug DNA was isolated per gram of leaf tissue. Some variation in DNA yield was found. Such variation might have been caused by leaf age. It was found that unopened, young leaf tissue (rolled) resulted in DNA with higher yield and better quality than fully open, mature leaf tissue. Using the same procedure, Rogers and Bendich (1988) reported DNA yields from 6 species in 6 different genera of 14-49 ug DNA per gram of leaf tissue, with some senescing leaf material giving a lower DNA yield.

The size of isolated DNA was determined by comparing it with *Hind*III-digested lambda DNA markers after agarose gel electrophoresis. Typically, the DNA size of the major part of a sample is larger than 23 kb (Fig. 23A). In Fig. 23A, the rapidly migrating bands represent RNA. If the major part of a particular DNA sample was below the 23 kb marker, this sample was not used for further DNA analysis.

DNA samples could be well digested by all restriction enzymes tested. Digested DNA samples on a gel (Fig. 23) revealed many distinct bands on a background smear. These bands represent either nuclear repeat DNA or ptDNA. DNA samples had a typical A_{260}/A_{280} value of between 1.7 and 2.0. Pure DNA preparations have A_{260}/A_{280} values of 2.0. Where there is contamination with either protein or phenol, the A_{260}/A_{280} value is significantly less than 2.0 (Sambrook *et al.* 1989).

Fig. 23. Total DNA isolated by CTAB method

- (A):** Undigested (1), *EcoRI*-digested (2) and *HindIII*-digested (3) leaf DNA separated on a 0.5% agarose gel.
- (B):** Four different leaf DNA samples digested with *EcoRI* and separated on a 0.8% agarose gel.

M 1 2 3

kb

23.1

9.4

6.6

2.3

2.0

A



kb

— 12.2

— 6.1

— 4.1

— 3.0

— 2.0

— 1.6

— 1.0

B

3.2. DNA Sequence differences between species

A dot blot was made using two-fold serially diluted DNA samples of *Z. elliotiana* (EL), *Z. rehmannii* (RE) and *Z. aethiopica* 'Childsiana' (CHI). The dot blot was hybridised (Section 2.6.2) with chemiluminescence-labelled total DNA of EL and CHI. In Fig. 24A, when total DNA of EL was used as a probe, the hybridisation signal with DNA of EL (lane 1) was stronger than those with DNA of RE and CHI (lane 2 and lane 3 respectively). The signals with DNA of RE and CHI were very similar. In Fig. 24B, when the same membrane was reprobed with chemiluminescence-labelled total DNA of CHI at the same hybridisation stringency condition, the signal was stronger with DNA of CHI (lane 3) than with DNA of EL and RE (lane 2 and lane 3 respectively). Again, the signals with DNA of EL and RE were similar. Thus, DNA samples of different species can be distinguished by this procedure,

Clear differences in DNA sequence between species was demonstrated in Fig. 24, although the hybridisation signals were not quantified. This clear difference in DNA sequence indicated that it would be possible to isolate species-specific DNA clones by differential screening of a partial genomic library with total DNA probes from different species.

3.3. Construction of a partial library

DNA of pGEM-3Z and CHI was completely digested with *Hind*III. A mixture of *Hind*III-digested DNA of pGEM-3Z and CHI was ligated and used to transform *E. coli* DH5 α (Section 2.2). The transformation results are summarised in Table 31. Ligation 1, which involved vector DNA without a phosphatase treatment, gave approximately 10% white colonies. Ligation 2, which involved vector DNA with a phosphatase treatment, gave a higher proportion (80%) of white colonies.

To determine size of inserts of clones in the library, miniprepations of plasmid DNA were made from 25 clones. Insert sizes were demonstrated to be from a few hundred base pair to 15 kb (Fig. 25).

Table 31. The results of *E. Coli* DH5α transformation

DNA	100 μl, LB/Amp/X-gal/IPTG ^a
No DNA	none
Uncut pGEM-3Z	many blue ^e
Ligation 1 ^b	65 white 600 blue
Ligation 2 ^c	500 white 120 blue
Ligation 3 ^d	0 white 105 blue

a: On each LB/Amp/X-gal/IPTG plate, 100 μl of transformed cells were plated. The white colonies contained recombinant plasmids and the blue colonies contained non-recombinant plasmids (see Section 2.2 for details).

b: Ligation 1 contained *Hind*III-digested, pGEM-3Z DNA and *Hind*III-digested, plant DNA.

c: Ligation 2 contained *Hind*III-digested, dephosphorylated, pGEM-3Z DNA and *Hind*III-digested, plant DNA.

d: Ligation 3 contained only *Hind*III-digested, dephosphorylated, pGEM-3Z DNA.

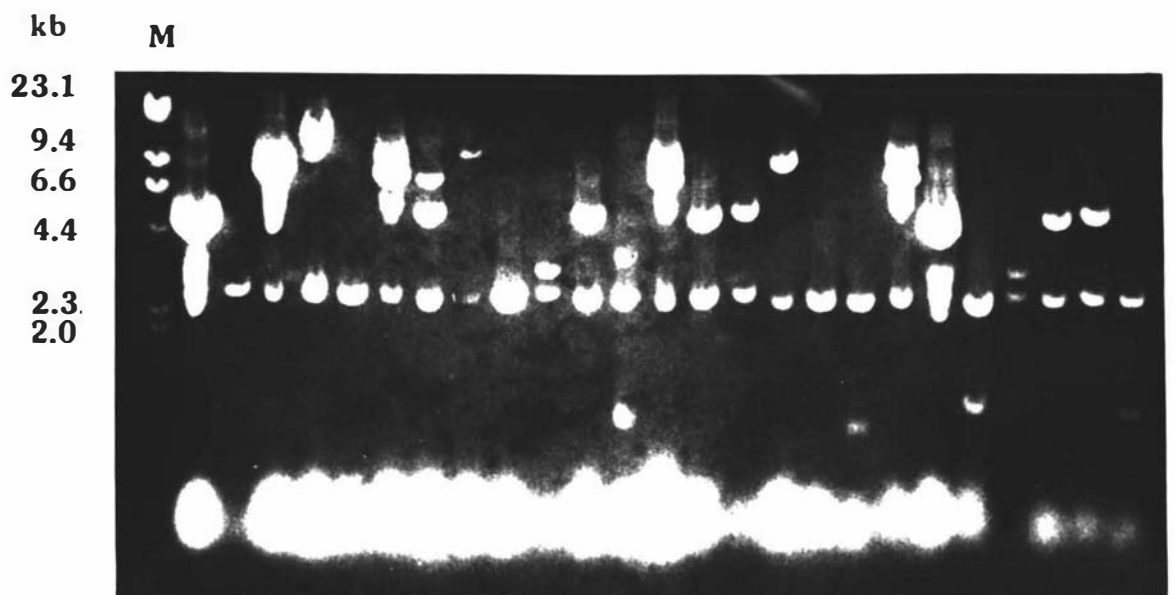
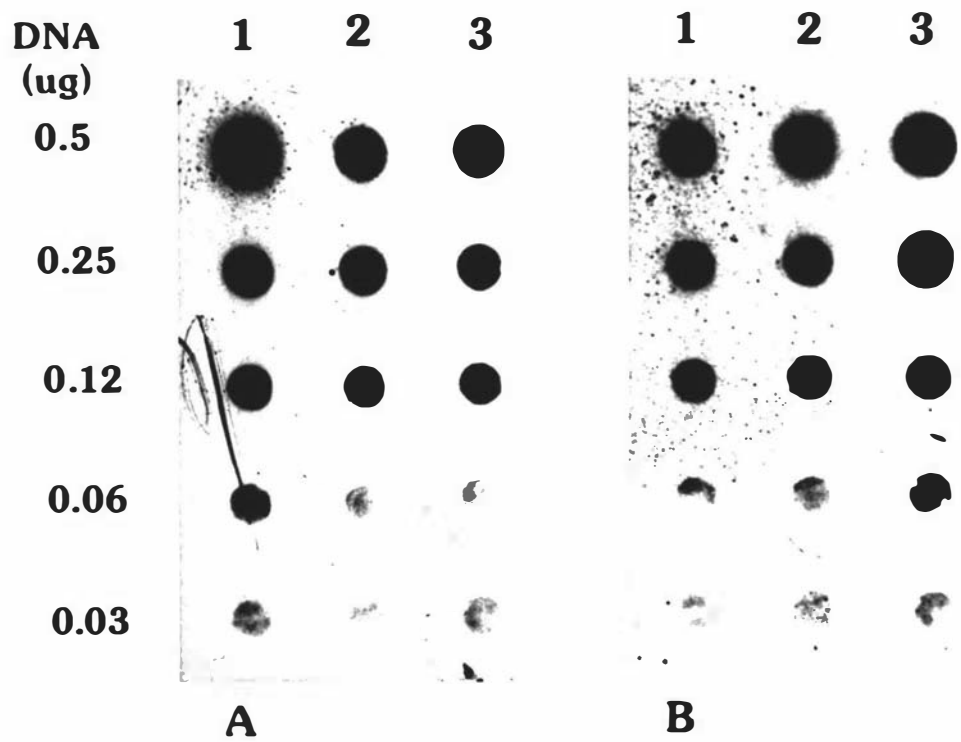
e: Too numerous to count. A 10⁻⁶ dilution plated without ampicillin yielded 160 clones.

Fig. 24. DNA sequence difference between species demonstrated on dot hybridisation

A two fold serially diluted DNA samples of *Z. elliotiana* (EL) (1), *Z. rehmannii* (RE) (2) and *Z. aethiopica* 'Childsiana' (CHI) (3) were blotted on to a nitrocellulose membrane. The membrane was probed with total DNA of EL (A) and reprobed with total DNA of CHI (B) (ECL system, 0.5 M NaCl in the hybridisation buffer).

Fig. 25. The size of insert DNA from different clones

The plasmid DNA of 25 clones was digested with *Hind*III and separated on a 0.7% agarose gel. *Hind*III-digested lambda DNA markers are on the left lane. A common 2.7 kb band represents the vector DNA in all 25 plasmids. The bands representing the insert DNA are different in size.



3.4. Isolation and characterisation of species-specific DNA clones

3.4.1. Isolation of species-specific DNA clones

Total DNA of different species was radioactively-labelled by the random primer extension technique (Section 2.5.1). The results of labelling were checked by PEI-cellulose thin layer chromatography (Section 2.5.1). In a typical result (Fig. 26), a very strong signal can be observed at the origin after autoradiography. This indicates that most of the ^{32}P -dCTP has been incorporated into DNA. Weak signals representing a small amount of unincorporated ^{32}P -dCTP and degraded ^{32}P -dCMP can also be observed.

Approximately 1500 clones on 6 blots were screened by colony hybridisation (Section 2.6.1) with radioactively-labelled, total DNA of CHI and EL. Approximately 20% of colonies showed a strong signal with both total DNA probes. Five clones showed a signal with the CHI probe but no signal with the EL probe. The hybridisation result of one blot is shown in Fig. 27. Hybridisation patterns are essentially the same with probes of CHI and EL. However, three clones (indicated by triangles) give no signal with the EL probe even though they give strong signals with the CHI probe. This type of clone was selected as being a putative species-specific clone.

Minipreparations of plasmid DNA were made from those five clones which showed differential signals between the CHI probe and the EL probe. Three did not show any insert DNA band after they were digested with *Hind*III and separated on a 0.8% agarose gel. It was possible that these clones contained an insert too small to be seen on a 0.8% agarose gel. These clones were not characterised further. One clone containing a 5.5 kb insert did not show differential signal on a Southern blot with DNA of different species and was also not characterised further. Another clone containing a 5.1 kb insert (Fig. 32) showed a differential signal with DNA of different species on a Southern blot (Fig. 28). This clone was named pZAC3.

Three bands, 1.5kb, 3.6kb, 5.1kb, were detected in CHI, *Z. aethiopica* (AE) and *Z. odorata* (OD), when a Southern blot of *Hind*III-digested, total DNA was probed with chemiluminescence-labelled, pZAC3 DNA (Fig. 28). The signal for OD was weaker even though the amount of DNA on the gel was the same. Possible reasons for the weaker signal include poor transfer of DNA from the gel to the membrane, lower copy number in OD and poor hybridisation or signal detection on the edge of the membrane. A

weak band about 2.7kb was found with DNA of 'Chromatella' (CHR). When Southern blots of the same CHR DNA preparation were probed with other probes containing pGEM-3Z DNA, a weak band at similar size was observed, such as in Fig. 33. In contrast, when Southern blots of another CHR DNA preparation were used, this band was not detected. It is possible that the first CHR DNA preparation was contaminated with a small amount of pGEM-3Z DNA. No hybridisation signals were detected in species of section II of the genus: *Z. pentlandii* (PE), *Z. rehmannii* (RE), *Z. elliotiana* (EL). The result in Fig. 28 demonstrated that the insert DNA sequences in clone pZAC3 was present specifically in species *Z. aethiopica* (CHI and AE) and *Z. odorata* (OD) but absent from plants in section II of the genus *Zantedeschia*.

Fig. 26. An autoradiograph of PEI-cellulose chromatogram

Radioactively-labelled (^{32}p -dCTP) DNA samples were applied at the origin of the chromatogram. ^{32}p -dCTP incorporated into DNA gave a strong radioactive signal at the origin. A small amount of unincorporated ^{32}p -dCTP and degraded ^{32}p -dCMP moved away from the origin and gave weak signals. The detail of the method was described in Section 2.5.1. (1) and (2) represent two different DNA samples.

Fig. 27. Selection of putative species-specific clones by colony hybridisation

A colony blot (nylon membrane) was probed with radioactively-labelled total DNA of *Z. aethiopica* 'Childsiana' (A) and reprobbed with radioactively-labelled total DNA of *Z. elliotiana* (B). Arrows indicate putative species-specific clones.

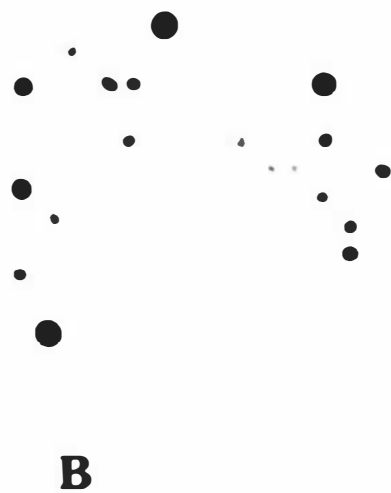
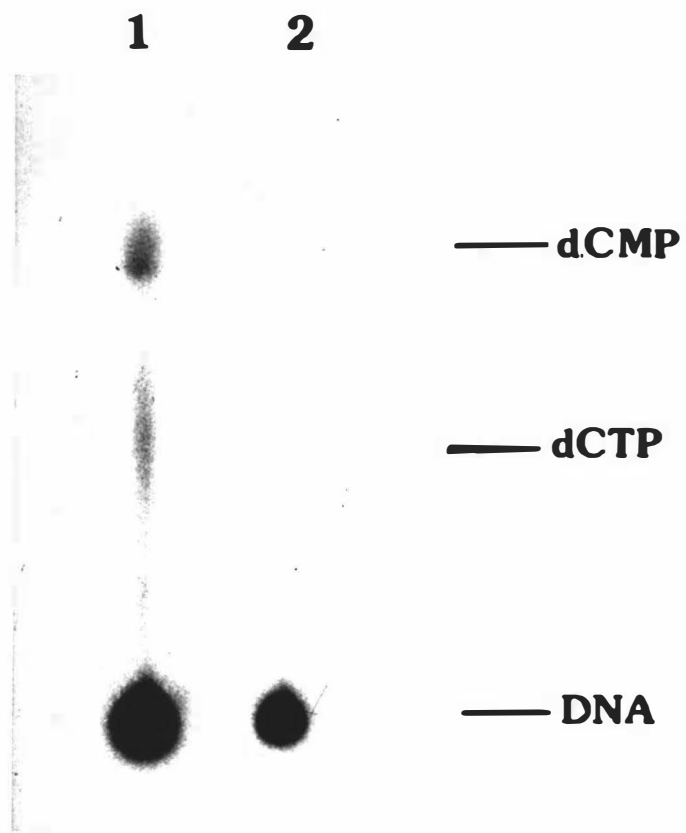
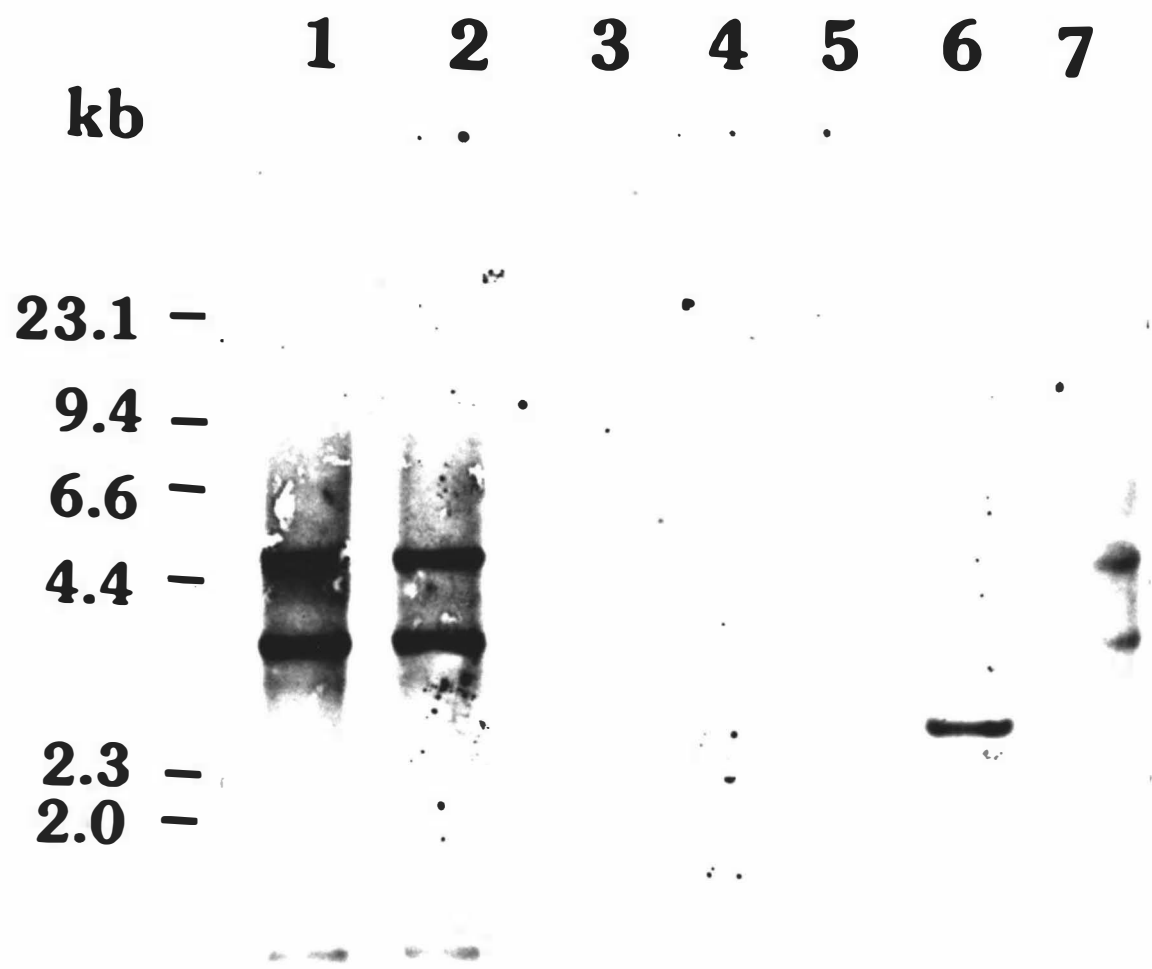


Fig. 28. Confirmation of species-specificity of pZAC3 on Southern blot

Total DNA of *Zantedeschia* plants was digested with *Hind*III. The resulting DNA fragments were separated on a 0.8% agarose gel and transferred to a nitrocellulose membrane. The membrane was probed with pZAC3. (ECL system, 0.3 M NaCl in the hybridisation buffer).

- (1): *Z. aethiopica* 'Childsiana' (CHI)
- (2): *Z. aethiopica* (AE)
- (3): *Z. pentlandii* (PE)
- (4): *Z. rehmannii* (RE)
- (5): *Z. elliottiana* (EL)
- (6): *Z.* 'Chromatella' (CHR)
- (7): *odorata* (OD)



3.4.2. Characterisation of the species-specific DNA clone pZAC3

Restriction enzyme map of pZAC3.

The cutting sites of *EcoRI*, *BglII* and *XbaI* were mapped in pZAC3 according to the method in Section 2.4. After restriction enzyme digestion and gel electrophoresis, the size of each restriction fragment was determined. Based on the sizes of these restriction fragments, the restriction-enzyme map was determined as in Fig. 29.

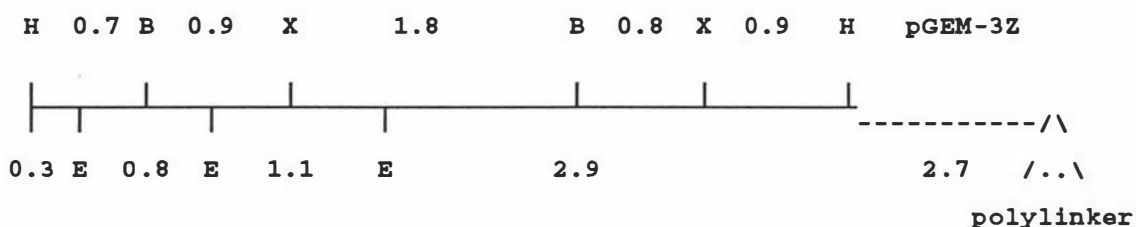


Fig. 29. Restriction enzyme map of pZAC3

H: *HindIII*, E: *EcoRI*, B: *BglII*, X: *XbaI*.

The numbers indicate fragment sizes in kilobases.

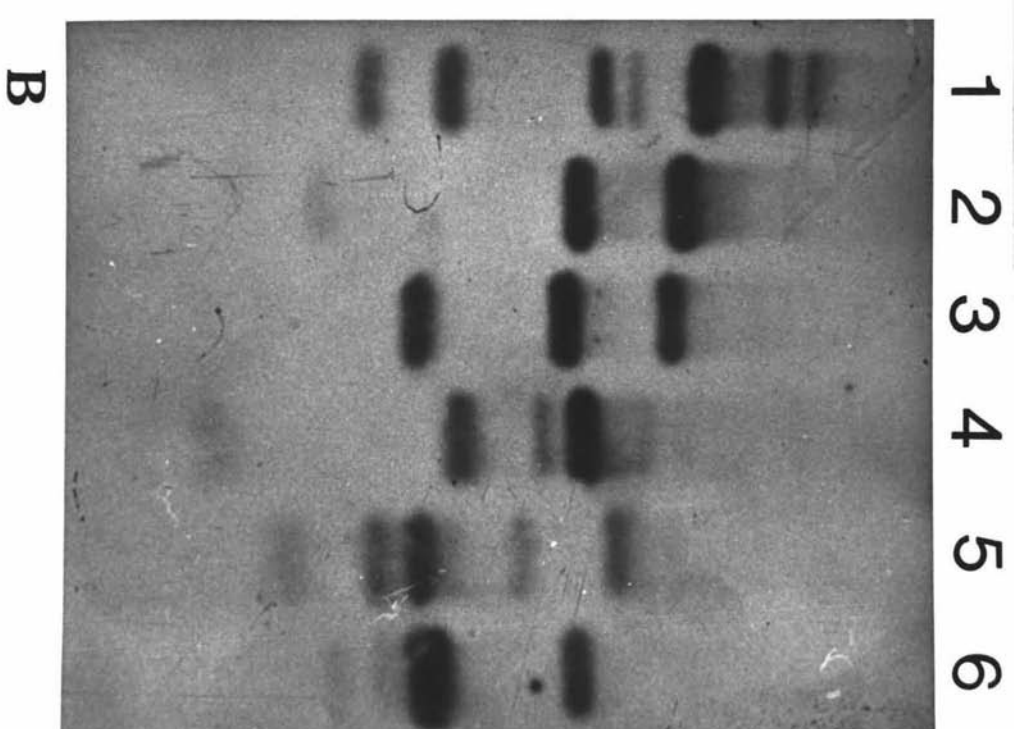
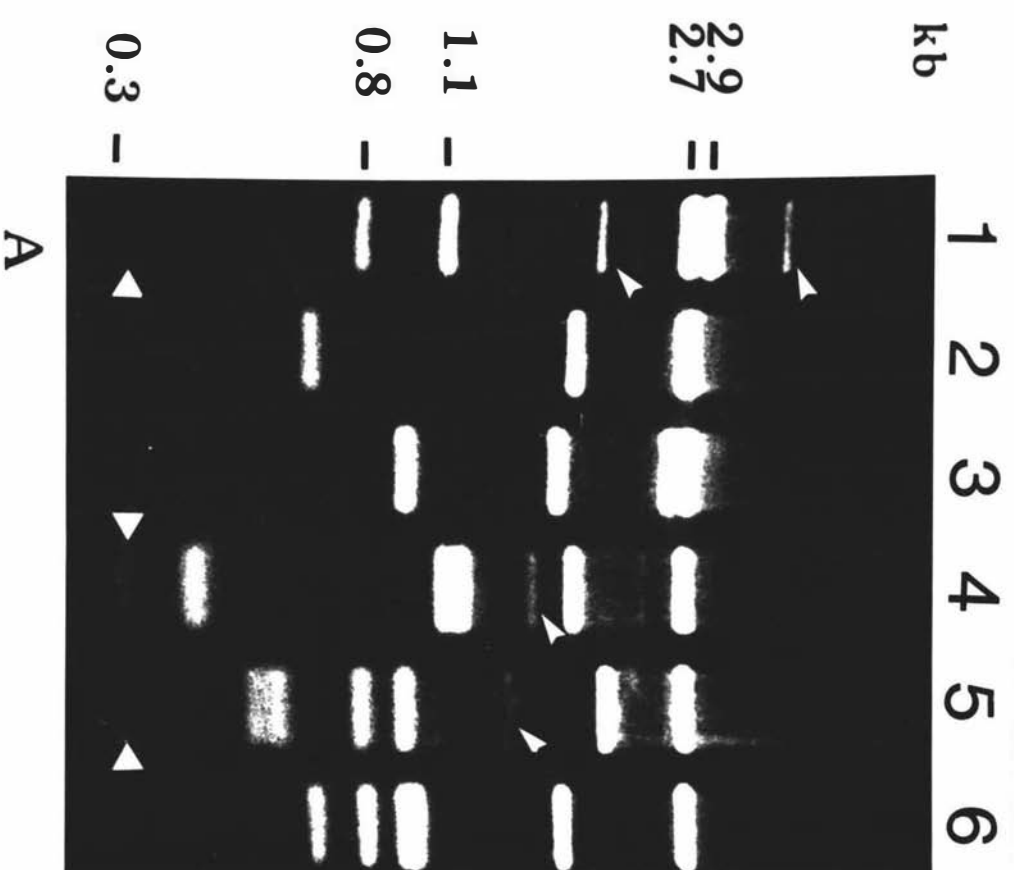
Different restriction fragments of pZAC3 with different copy number

On a Southern blot, DNA fragments of pZAC3 (after digestion with *HindIII*, *EcoRI*, *BglII* and *XbaI*) showed different signal intensities after they were hybridised to radioactively-labelled, total, CHI DNA. Some fragments had strong signals and other fragments had weak signals (Fig. 30). Fragments giving a strong signal are indicated in Fig. 31. It was found that only two fragments, 0.9 kb *BglII/XbaI* fragment (named repeat A) and 0.9 kb *HindIII/XbaI* fragment (named repeat B), or other fragments containing either a whole or a part of repeat A or repeat B had a strong signal. It is concluded, therefore, that these two 0.9 kb fragments have a higher copy number than the rest of the fragments in pZAC3.

Fig. 30. Different restriction fragments of pZAC3 showing different hybridisation signals with a total-DNA probe of *Z. aethiopica* 'Childsiana'

- (A):** Plasmid DNA of pZAC3 was digested with *Hind*III and then with different restriction enzymes (see 1 to 6). The resulted DNA fragments were separated on a 1.0% gel.
- (B):** A Southern blot (nylon membrane) was made from the gel in (A) and probed with radioactively-labelled, total DNA of *Z. aethiopica* 'Childsiana'. The arrows indicate incompletely digested DNA fragments and the triangles indicate a weak 0.3 kb band.

- (1): *Eco*RI
(2): *Bgl*II
(3): *Xba*I
(4): *Eco*RI + *Bgl*II
(5): *Eco*RI + *Xba*I
(6): *Bgl*II + *Xba*I



Enzymes

Used

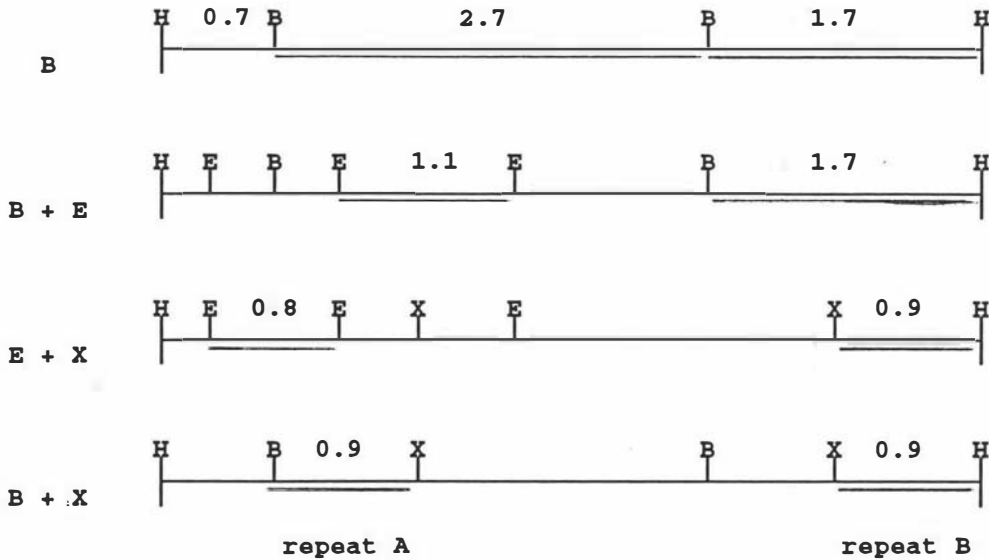


Fig. 31. The map of restriction fragments of pZAC3 showing strong signals with a total-DNA probe of *Z. aethiopica* 'Childsiana'

Summary of the results in Fig. 30. Strong signal regions are indicated by a double line.

The two fragments with higher copy number are different

The 1.6 *Hind*III/*Xba*I fragment which contained repeat A, was purified from low melting temperature gel (Section 2.3.3). The purified 1.6 kb DNA fragment was chemiluminescence-labelled and hybridised to the same blots in Fig. 30. On the *Hind*III/*Xba*I digested lane, this 1.6 kb fragment only hybridised to itself. On other lanes, it only hybridised to the fragments which contained a whole or a part of this 1.6 kb fragment (figure not shown). Therefore, the repeat A had no homology with the repeat B. Also, there was no homology between this 1.6 kb fragment and the rest of pZAC3 DNA.

3.5. Selection of clones for RFLP analyses in *Zantedeschia*

RFLP analyses in *Zantedeschia* with clones from the CHI library

Two clones, pZAC1 and pZAC2, which showed strong signals in colony hybridisation gave RFLPs on Southern blots with DNA of different *Zantedeschia* species.

pZAC1. Digestion of pZAC1 with *Hind*III produced a 2.5 kb insert DNA and a 2.7 kb vector DNA (Fig. 32). Against a Southern blot of *Hind*III-digested, total DNA of *Zantedeschia*, pZAC1 hybridised with a 2.5 kb band in *Z. aethiopica* and a 2.2 kb band in *Z. elliottiana*, *Z. rehmannii*, *Z. 'Chromatella'*, *Z. pentlandii* (Fig. 33), and *Z. odorata* (data not shown). Several additional weak bands were also observed. The sizes of these weak bands were larger than 2.5 kb in every experiment. However, intensity and position varied from experiment to experiment. These weak bands may be present for the related DNA sequences to the 2.5 kb insert DNA of pZAC1, or incompletely digested DNA fragments. One of these bands was about 2.7 kb in 'Chromatella' (Fig. 33). This 2.7 kb band was assumed to be pGEM-3Z DNA. The 2.5 kb and 2.2 kb bands were stable although these weak bands varied. Against a Southern blot of *Eco*RI-digested total DNA of *Zantedeschia*, pZAC1 hybridised with a 2.8 kb band in *Z. aethiopica* and a 2.5 kb band in other species (data not shown).

pZAC2. pZAC2 contained a 12.1 kb insert DNA which can be cut into 6 fragments (0.6, 0.7, 1.5, 1.7, 3.3 and 4.3 kb) by *Eco*RI (Fig. 32). It hybridised with a 12.1 kb *Hind*III fragment of *Z. aethiopica* (AE, CHI, AE_{sp}) and *Z. odorata* (OD), and with 3 *Hind*III fragments (1.0, 4.7 and 5.7 kb) of plants in section II of the genus on a Southern blot (Fig. 34A). The 1 kb *Hind*III band is faint in Fig. 34A but it was clearly seen on the original x-ray film. On a Southern blot of *Eco*RI-digested leaf DNA, pZAC2 revealed seven bands (0.6, 0.7, 1.5, 3.5, 4.0, 4.3, 4.8 kb) for CHI (Fig. 34B). The 0.6 and 0.7 kb bands are not clearly seen in Fig. 34B but were clearly showed in other Southern blots. AE had a 1.4 kb band instead of a 1.5 kb band as compared with CHI (Fig. 34B). AE_{sp} and OD showed the same or similar band pattern to AE but *Z. elliottiana* had a different band pattern (Table 32).

Fig. 32. The size of the insert DNA of pZAC1, pZAC2 and pZAC3

pZAC1 (1), pZAC2 (2), and pZAC3 (3) were digested with *Hind*III and pZAC2 was digested with *Hind*III/*Eco*RI (4). Digested DNA fragments were separated on a 0.8% agarose gel. Lambda DNA size markers are shown on both sides. A common 2.7 kb band represents the vector DNA in all three plasmids.

Fig. 33. RFLP analysis with pZAC1

*Hind*III-digested total DNA of *Zantedeschia* plants was separated on a 0.8% agarose gel and transferred to a nylon membrane. The membrane was probed with pZAC1 (ECL system, 0.3 M NaCl in the hybridisation buffer). Lambda DNA size markers are shown on the left.

(1): *Z. aethiopica* 'Childsiana'

(2): *Z. aethiopica* (AE)

(3): *Z. elliottiana*

(4): *Z. rehmannii*

(5): *Z. 'Chromatella*

(6): *Z. pentlandii*

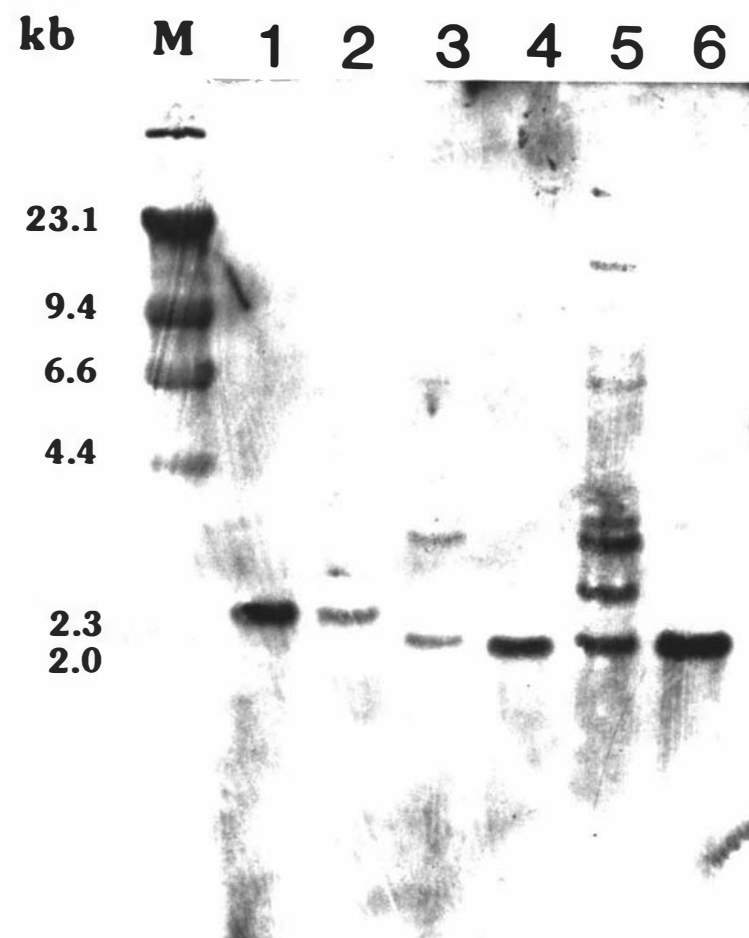
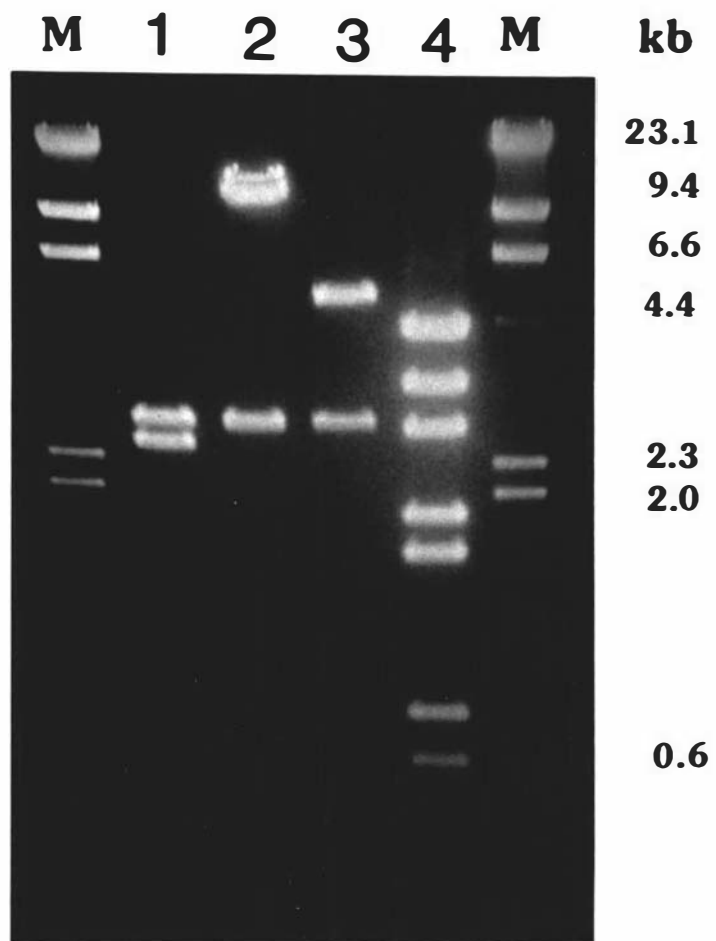


Fig. 34. RFLP analysis with pZAC2

Zantedeschia DNA was digested with a restriction enzyme. The resulting DNA fragments were separated on a 0.8% agarose gel and transferred to a nitrocellulose membrane. The membrane was probed with pZAC2 (ECL system, 0.3 M NaCl in the hybridisation buffer).

(A): *Hind*III digestion

(B): *Eco*RI digestion

(1): *Z. aethiopica* (AE)

(5): *Z. elliottiana* (EL)

(2): *Z. aethiopica* 'Childsiana' (CHI)

(6): *Z. rehmannii* (RE)

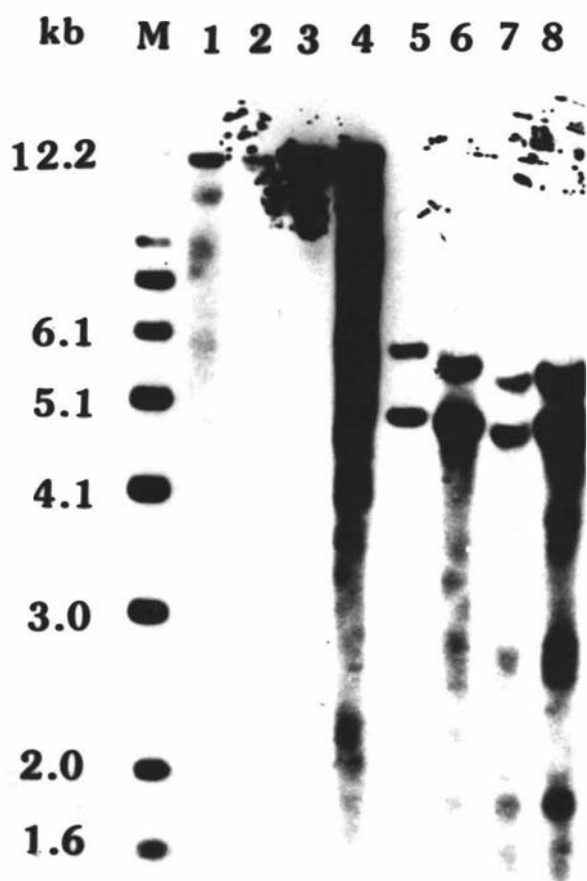
(3): *Z. aethiopica* (AE_{sp})

(7): *Z. pentlandii* (PE)

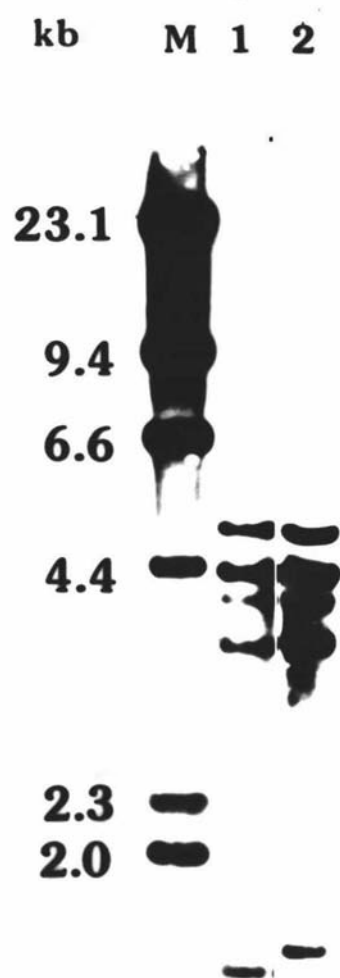
(4): *Z. odorata* (OD)

(8): *Z. 'Chromatella* (CHR)

(M): 1 kb DNA markers in (A) and *Hind*III-digested DNA markers in (B).



A



B

RFLP analyses in *Zantedeschia* with 18s rDNA of apple

A Southern blot of restriction-enzyme-digested, total DNA of CHI, OD, CHR and EL was probed with 18s rDNA of apple (Fig. 35). On *EcoRI* digestion, all four species showed a common band at 2.0 kb. CHR and EL had two common bands at 4.0 kb and 5.0 kb, which were not present in CHI or OD. On the other hand, CHI and OD had a common band at 3.7 kb, which was not present in CHR or EL. Furthermore, CHI had a specific band at 4.7 kb and OD had a specific band at 6.0 kb. Upon *HindIII* digestion, CHI, CHR and EL showed a common band of high molecular weight, which was weak or absent in OD. CHI and OD showed a common band at 5.4 kb, which was not present in CHR or EL whereas, CHR and EL had a common band at 5.7 kb, which was not present in CHI or OD. The banding pattern produced by *BglII* was similar to that of *HindIII* digestion, except that the band sizes were different (Fig. 35).

The results in Fig. 35 show that a probe of 18s rDNA can distinguish between CHI and OD, as well as between CHI and CHR or EL, between OD and CHR or EL. There are no different DNA bands between CHR and EL evident in Fig. 35. On another Southern blot of *HindIII*-digested DNA of CHR, EL, RE (*Z. rehmannii*) and PL (*Z. pentlandii*), no different DNA bands were detected. It is not known whether different DNA bands can be detected among these species in section II of the genus with further restriction enzymes.

Fig. 35. RFLP analysis with apple 18s rDNA

After restriction enzyme digestion, DNA fragments were separated on a 0.8% agarose gel and transferred to a nitrocellulose membrane. The membrane was probed with apple 18s rDNA (ECL system, 0.3 M NaCl in the hybridisation buffer).

(1) to (4): *Eco*RI digestions of CHI, OD, CHR and EL respectively.

(5) to (8): *Hind*III digestions of CHI, OD, CHR and EL respectively.

(9) to (12): *Bgl*II digestions of CHI, OD, CHR and EL respectively.

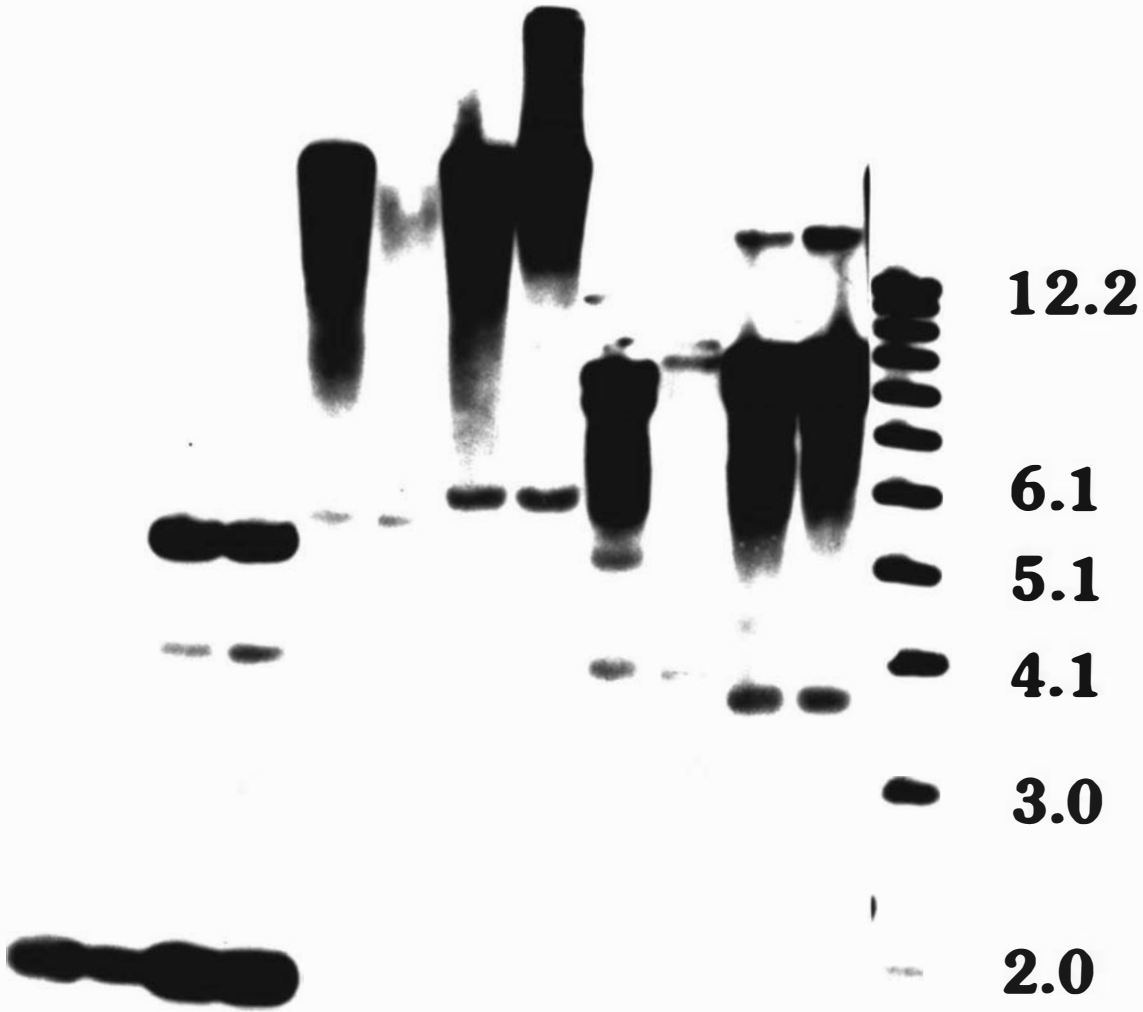
CHI: *Z. aethiopica* 'Childsiana'

OD: *Z. odorata*

CHR: *Z. 'Chromatella'*

EL: *Z. elliottiana*

1 2 3 4 5 6 7 8 9 10 11 12 M



3.6. Isolation and characterisation of ptDNA clones

Selection of putative ptDNA clones

According to the method in Section 2.7.1, a clone containing only pGEM-3Z did not show a signal on one dot blot and only a very weak signal on another blot. Other clones showed a wide range of signal intensity variation. Poor DNA purity and poor uniformity of DNA amount on the blots were the probable reasons for this variation. DNA samples made by the miniprep method contain protein and cell debris which may bind non-specifically to the probe in the ECL system. The same volume of plasmid DNA preparation from different clones was used on the blots. However, the DNA concentration of different plasmid preparation may have been different. Nevertheless, forty out of seventy clones showed medium to strong hybridisation signals and were considered to be putative ptDNA clones. pZAC1, pZAC2 and pZAC3 were included in one of the two dot blots. pZAC1 and pZAC2 showed strong signal whereas pZAC3 did not show any signal.

Confirmation of ptDNA clones

Using the method described in Section 2.7.2, pZAC1 and pZAC2 showed biparental bands on some hybrids and only maternal bands on other hybrids. It is therefore concluded that they are ptDNA clones as nuclear DNA clones would show biparental bands for all hybrids. The results for pZAC1 are shown in Figs. 43 and 44.

The plastid origin of another four clones (No.46, No.73, No.76 and No.95) was supported in a similar way. Probes made from these clones gave biparental DNA bands in hybrid No.43 (embryo origin) whereas they gave only maternal DNA bands in hybrid No.47 (haustorial origin) (Fig 36). Nuclear DNA clones would show biparental bands on hybrid No.47 also. With probes of clones No.76 and No.95, hybrid No.47 showed two parental bands as well as one or two extra bands. The reason for this is not known.

These Southern hybridisations also demonstrate that any one of the four clones can distinguish CHI from CHR and OD (Fig. 36 A, B, C, D). The sizes of the DNA bands in Fig. 36 are summarised in Table 32. The sizes of inserts of clone No.46, clone No.73, clone No.76 and clone No. 95 are 2.5 kb, 3.3 kb, 5.5 kb and 5.4 kb respectively.

The ptDNA in clone No.95 appears to be at an end of the inverted repeats as it hybridises with two DNA fragments of different size.

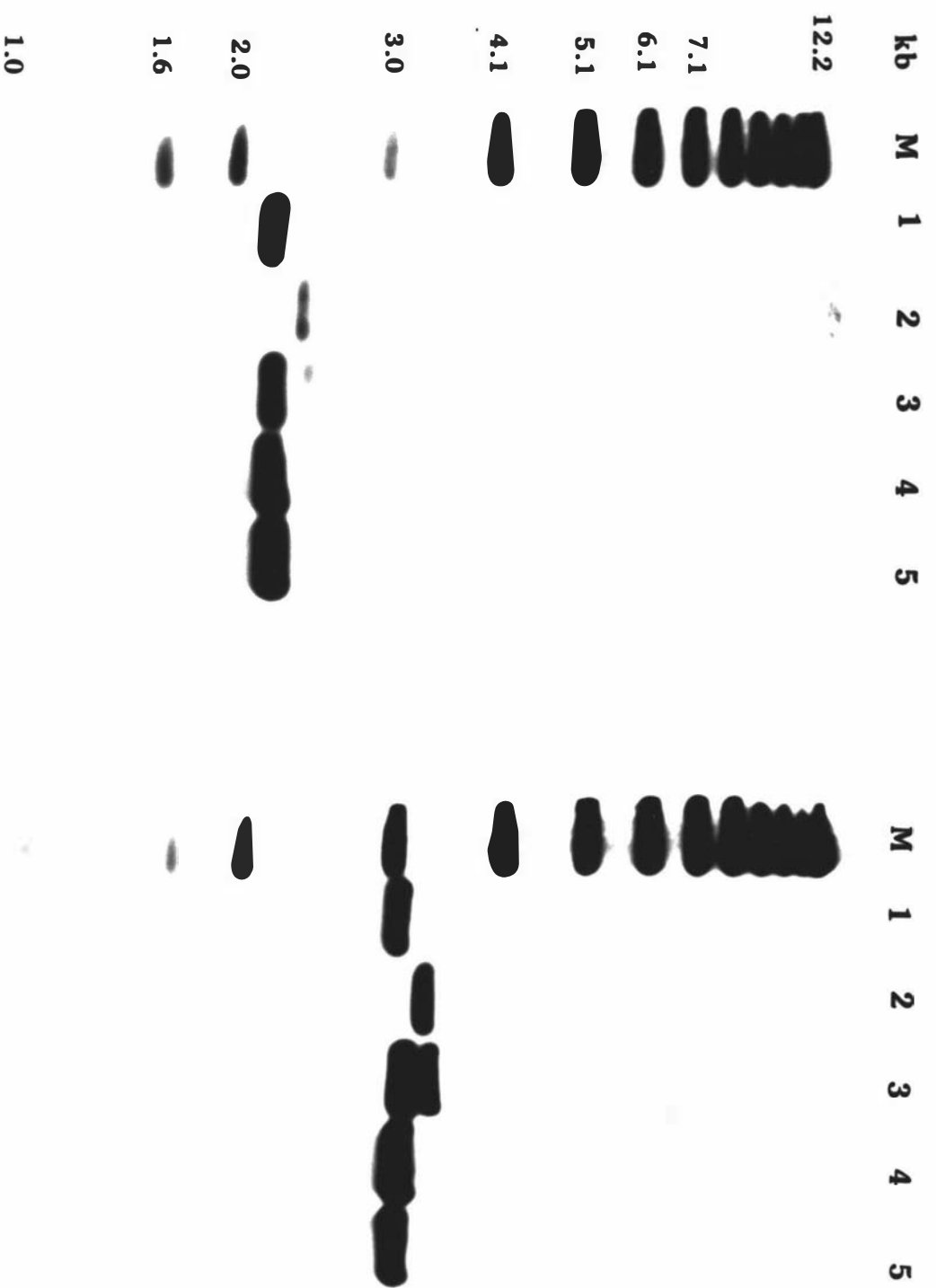
Physical position of the inserts in pZAC1 and pZAC2 in the plastome

Against a dot blot (Section 2.7.3), pZAC1 and pZAC2 showed visible hybridisation to DNA from leaf and root of CHI (Fig. 37). The hybridisation to the root was weaker than that to the leaf, presumably because the root contained a lower number of plastids than the leaf. pZAC1 and pZAC2 only showed very weak hybridisation (background) to lambda DNA (Fig. 37). pZAC1 hybridised to K6 and K15 strongly and to K19, K41 and K42 weakly (Fig. 37A). pZAC2 hybridised to K41 and K42 strongly and to K6 and K19 weakly (Fig. 37B). As most higher plants have a similar plastome structure and plastid-gene order (Palmer 1990), pZAC1 and pZAC2 were mapped in the plastome (Fig. 38) on the basis of the physical map of kiwifruit plastome and the dot-hybridisation results. The reason for the weak signals of these two clones to K19, K41 and K42, or to K6 and K19, is possibly partial sequence homology. On a Southern blot, pZAC1 hybridised weakly to a 1.7 kb *EcoRI* band of pZAC2.

Fig. 36. Confirmation of ptDNA clones by RFLP analysis

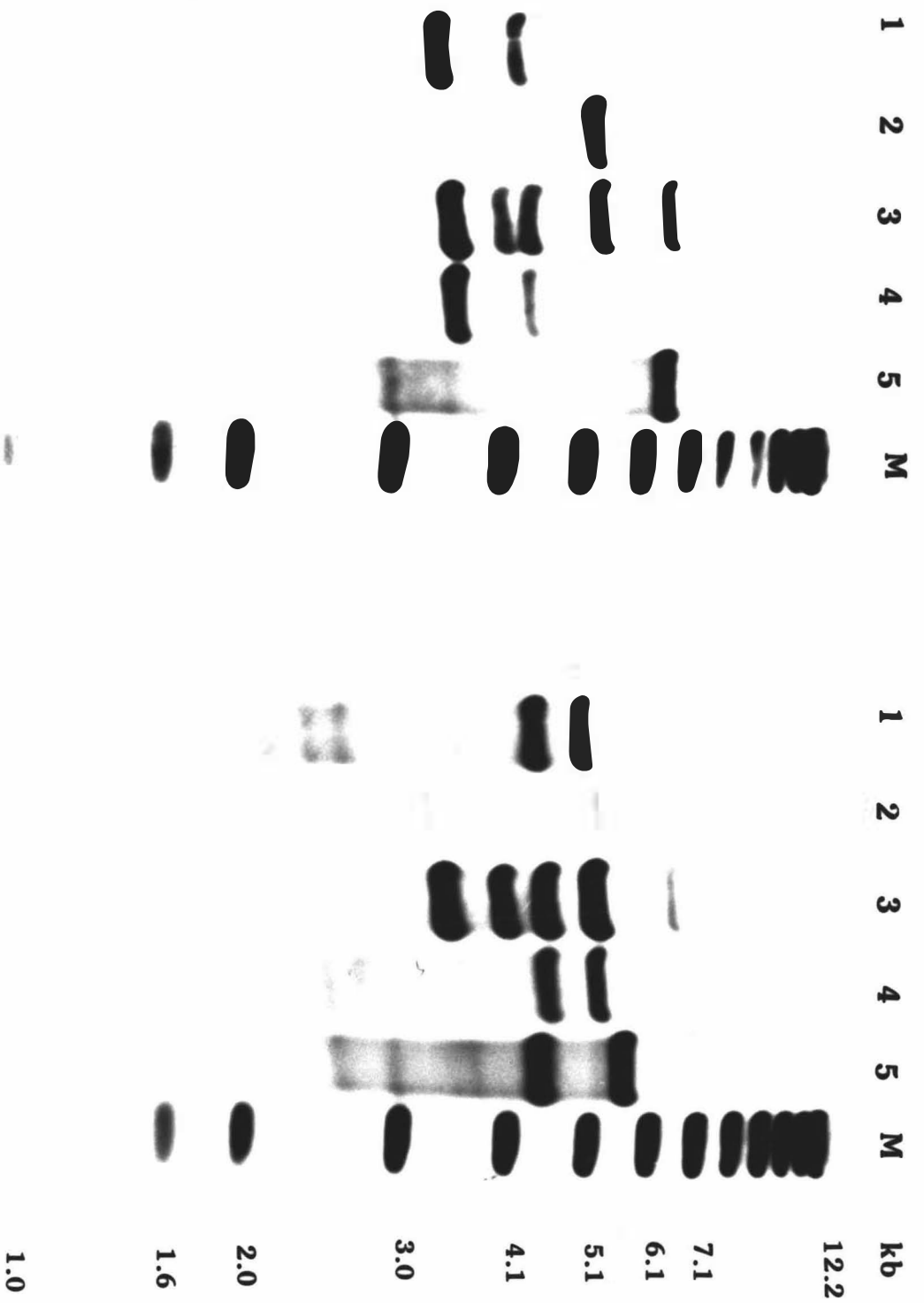
Total DNA of *Zantedeschia* plants was digested with *Hind*III. The resulting DNA fragments were separated in duplicate lanes on a 0.8% agarose gel and transferred to a nitrocellulose membrane. The membrane was cut into two pieces. One piece was probed with clone No.46 (A) and reprobed with clone No.73 (B). The other piece was probed with clone No.76 (C) and reprobed with clone No.95 (D). The ECL system was used for the hybridisation and 0.3 M NaCl was used in the hybridisation buffer.

- (1): *Z. 'Chromatella'* (CHR)
- (2): *Z. aethiopica 'Childsiana'* (CHI)
- (3): hybrid No.43 (embryo origin) of CHR X CHI
- (4): hybrid No.47 (haustorial origin) of CHR X CHI
- (5): *Z. odorata* (OD)

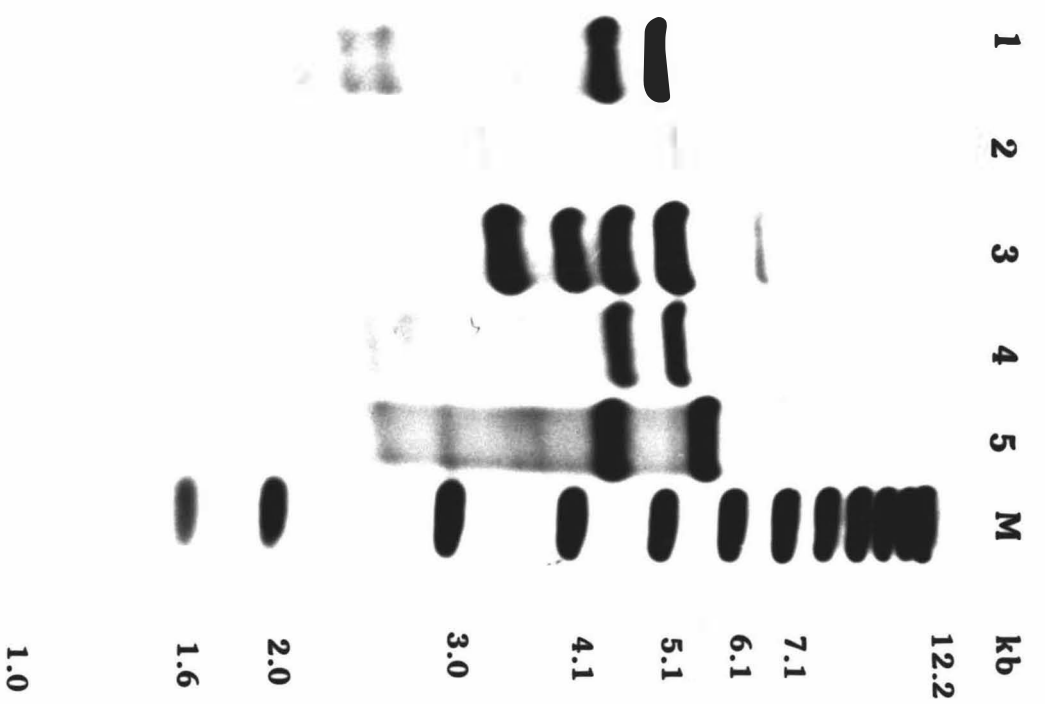


A

B



C



D

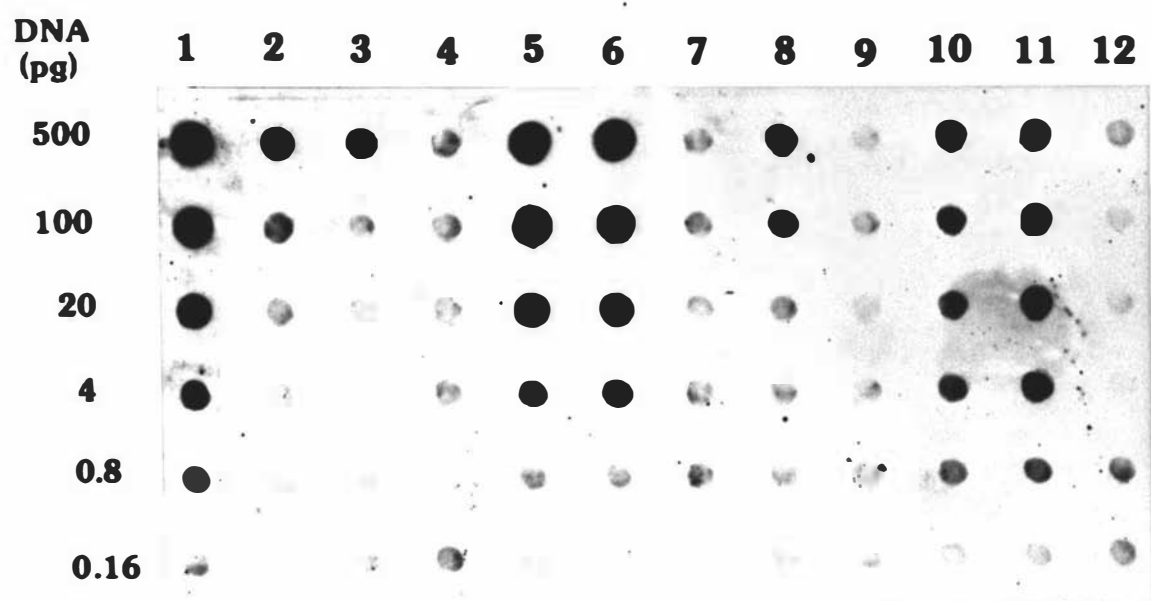
Fig. 37. Hybridisation of pZAC1 and pZAC2 to kiwifruit ptDNA clones on a dot blot

A dot blot (nitrocellulose membrane) was made with serially diluted DNA samples. The blot was probed with pZAC1 (A) and reprobed with pZAC2 (B) (ECL system, 0.3 M NaCl in the hybridisation buffer).

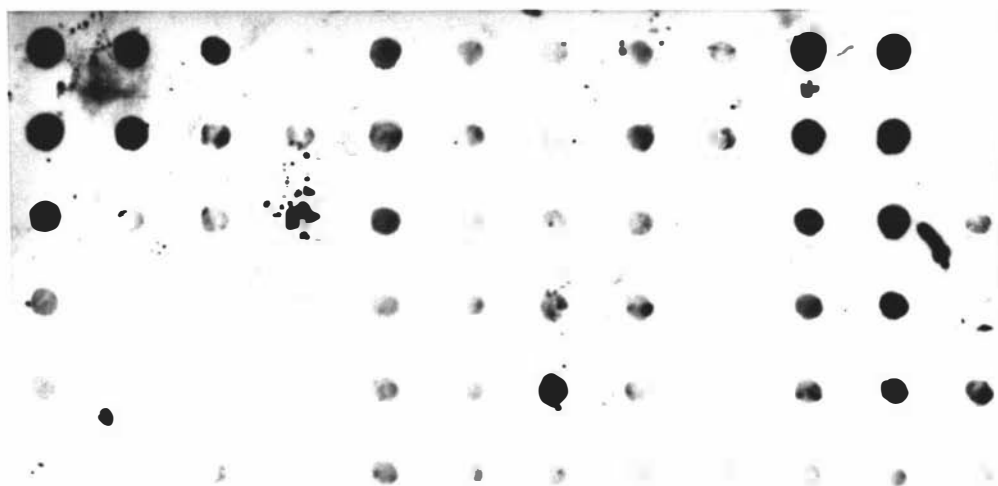
- | | |
|------------------------------|--------------|
| (1): pZAC1 | (7): K17 |
| (2): total leaf DNA of (CHI) | (8): K19 |
| (3): total root DNA of CHI | (9): K24 |
| (4): K4, | (10): K41 |
| (5): K6 | (11): K42 |
| (6): K15, | (12): lambda |

CHI = *Z. aethiopica* 'Childsiana'

K4 = λ kiwi4, K6 = λ kiwi6, etc.



A



B



Fig. 38. The location of pZAC1 and pZAC2 on the plastome

The upper part of this figure is the physical map of the kiwifruit plastome with the location of ten kiwifruit ptDNA clones (42 = λ kiwi42, 6 = λ kiwi6, etc.) indicated. The filled bars represent the minimum size of the inverted repeat and the open bars indicate the maximum size of the inverted repeat (following Crowhurst *et al.* 1990).

The location of pZAC1 and pZAC2, shown at the lower part of this figure, was determined according to the hybridisation result in Fig. 37.

4. Discussion

In this Chapter, the construction of a partial library with total leaf DNA of CHI is described. From the library, a species-specific DNA clone was isolated. This clone has been used to identify hybrids produced from crosses between *Z. aethiopica* (as the paternal parent) and section II of the genus (Chapter 9). To identify hybrids in other crosses, DNA clones which gave RFLP polymorphisms between species were sought. For hybrid identification, repetitive DNA clones are preferred because they can detect multi-loci in a genome and make the DNA detection much easier than single copy DNA clones. Two clones, pZAC1 and pZAC2 were used to detect RFLP polymorphisms between species and appeared initially to be repetitive DNA clones. Subsequently, however, these two clones were identified as ptDNA clones. At this stage, heterologous rDNA clones were used to characterise the nuclear genomes of different species and hybrids. It was found that the apple 18S rDNA could be used to distinguish different species and identify hybrids between these species by RFLP analysis.

Several approaches for species-specific clone isolation were reviewed in Chapter 3. This study attempted to clone species-specific DNA from distinct DNA bands. If a prominent band had been observed in one species but not in another, species-specific repetitive DNA sequence would have been simply cloned from this band (Schweizer *et al.* 1988). However, such a band was not observed after restriction enzyme-digested DNA of *Zantedeschia* species was separated on an agarose gel. Clear differences in DNA sequence between *Zantedeschia* species were observed by dot hybridisation (Fig. 24). This indicated that it would be possible to isolate species-specific DNA sequences by screening a partial genomic library with heterogeneous genomic probes. On colony hybridisations, 5 of 1500 clones showed differential signals initially with radioactively-labelled total DNA of different species. The species specificity of one clone, pZAC3, was confirmed by Southern hybridisation.

pZAC3 is believed to be a nuclear DNA clone. The insert of pZAC3, a 5.1 kb *Hind*III fragment, did not show hybridisation to 8 clones of kiwifruit ptDNA. pZAC3 hybridised with three *Hind*III bands (5.1, 3.6 and 1.5 kb) of CHI and OD on a Southern blot (Fig. 28) and different parts of the insert in pZAC3 had different copy number (Fig. 30). An organelle clone would have given one band or two bands. Furthermore, all hybrids tested contained pZAC3 sequence inherited from the paternal parent. Organelle DNA would have showed a maternal or predominantly maternal inheritance. All these features support the nuclear origin of pZAC3.

Heterologous rDNA probes have recently been used for hybrid identification (Honda & Hirai 1990). In the present study, a 1.5 kb *EcoRI/XbaI* fragment derived from the 18s rDNA of apple hybridised strongly with a 2.0 kb *EcoRI* fragment in all *Zantedeschia* species and weakly with another two *EcoRI* bands which varied in size in different species (Fig. 35). The 2.0 kb DNA fragment is possibly within the 18s coding region or it may overlap with the adjacent 25s region. This would account for the strong hybridisation signal and no polymorphisms between species. In the other two larger bands for each species, the 18s rDNA may overlap into the intergenic spacer region of the rDNA which exhibits polymorphisms between species. In the genus *Quercus*, there is an identical restriction map within the 18s and 25s region but each species has several rDNA repeats which differ in the intergenic spacer length (Bellarosa *et al.* 1990).

The DNA samples which were prepared from *Zantedeschia* plants by the CTAB method (Rogers and Bendich 1988) were suitable for Southern analysis and DNA cloning. Compared to cesium chloride gradient centrifugation, the CTAB method is simple and fast. Even though only one chloroform extraction was performed in some DNA preparation experiments, DNA samples were pure enough for restriction enzyme digestion. These DNA samples include total cellular DNA. Following restriction enzyme digestion, approximately 30 distinct bands on a background smear could be observed on an ethidium bromide stained gel (Fig. 23). These bands may represent highly repetitive nuclear DNA but some bands may represent ptDNA. In green tissue of wheat, approximately 10% of the cellular DNA is ptDNA and there are about 14,000 copies of plastome per haploid nuclear genome (Day and Ellis 1984). In suspension cultures of tobacco, green cells contain approximately 9,500-12,000 copies of plastome per cell (Lannon *et al.* 1985).

An objective of the present study was to characterise the hybrid plastomes using cloned ptDNA as probes when the interspecific hybrids (described in Chapter 6 and Chapter 7) were albino, virescent or chimeric. As up to 10% of leaf DNA may be ptDNA, the CHI library, which was constructed with total leaf DNA, should contain many ptDNA clones. Of 70 clones which showed strong colony hybridisation signals with total DNA probes, 40 clones were hybridised to a mixture of eight ptDNA clones of kiwifruit and considered to be putative ptDNA clones. Six of these clones (pZAC1, pZAC2, No.46, No.73, No.76 and No.95) were hybridised to Southern blots of DNA from two albino hybrids. The plastid origin of these six clones was also demonstrated

since they showed only maternal bands for a hybrid and both parental bands for the other hybrid. If any clone had been nuclear DNA, it would have shown both parental bands for two hybrids. These ptDNA clones are very useful for characterisation of hybrid plastomes as they can demonstrate RFLP polymorphisms between species (Table 32).

In recent years, ptDNA (Palmer 1987, Palmer *et al.* 1988), rDNA (Jorgensen & Cluster 1988, Govindaraju *et al.* 1992) and other repetitive DNA (Dvorak & Zhang 1990) analyses have been widely used in phylogenetic studies. The Southern hybridisation results in this study, summarised in Table 32, gave the first molecular data on the relationships between *Zantedeschia* species. All plant materials tested in section II of the genus showed the same band pattern. All three genotypes of *Z. aethiopica* (section I of the genus) showed the same band pattern, except a 1.5 kb *EcoRI* band in CHI and a 1.4 kb band in AE and AEsp when probed with pZAC2. Although some bands were common to both sections, other bands were distinctive to one section only. These results imply that plants in each section of the genus are closely related and that the two sections are distinct. This implication agrees well with the morphological classification (Letty 1973) and the sexual hybridisation results in Chapter 6. The newly described species, *Z. odorata* (OD), was placed between the previously described two sections on the basis of morphological characters (Perry 1989). In the Southern hybridisation results, OD showed some bands common with *Z. aethiopica*, some bands common with section II and its own specific bands which are not present in either of the two sections. These results indicate that OD is distinct from the previously described two sections and falls between the two sections. This indication is compatible with the data of chromosome analysis in Chapter 4 and with the data of interspecific hybridisation in Chapter 7.

Table 32. RFLP bands (size in kb) showing significances of species relationships in *Zantedeschia* ^a

Probe	Enzyme	section I			OD	section II				Fig.
		AE	AE _{sp}	CHI		CHR	RE	PL	EL	
rDNA	<i>EcoRI</i>	<u>4.7</u>	<u>4.7</u>	<u>4.7</u>	<u>6.0</u>	<u>5.0</u>	<u>5.0</u>	<u>5.0</u>	<u>5.0</u>	35
		<u>3.6</u>	<u>3.6</u>	<u>3.6</u>	<u>3.6</u>	<u>4.0</u>	<u>4.0</u>	<u>4.0</u>	<u>4.0</u>	
		<u>2.0</u>	<u>2.0</u>	<u>2.0</u>	<u>2.0</u>	<u>2.0</u>	<u>2.0</u>	<u>2.0</u>	<u>2.0</u>	
pZAC1	<i>HindIII</i>	<u>2.5</u>	<u>2.5</u>	<u>2.5</u>	<u>2.2</u>	<u>2.2</u>	<u>2.2</u>	<u>2.2</u>	<u>2.2</u>	33
	<i>EcoRI</i>	<u>2.8</u>	<u>2.8</u>	<u>2.8</u>	<u>2.5</u>	<u>2.5</u>	<u>2.5</u>	<u>2.5</u>	<u>2.5</u>	
pZAC2	<i>HindIII</i>	<u>12.1</u>	<u>12.1</u>	<u>12.1</u>	<u>12.1</u>	<u>5.7</u>	<u>5.7</u>	<u>5.7</u>	<u>5.7</u>	34
						<u>4.7</u>	<u>4.7</u>	<u>4.7</u>	<u>4.7</u>	
						<u>1.0</u>	<u>1.0</u>	<u>1.0</u>	<u>1.0</u>	
	<i>EcoRI</i>	<u>4.8</u>	<u>4.8</u>	<u>4.8</u>	<u>4.8</u>	-	-	-	<u>9.5</u>	34 ^b
		<u>4.3</u>	<u>4.3</u>	<u>4.3</u>	<u>4.3</u>					
		<u>4.0</u>	<u>4.0</u>	<u>4.0</u>	<u>4.0</u>				<u>4.0</u>	
		<u>3.5</u>	<u>3.5</u>	<u>3.5</u>	<u>3.2</u>				<u>3.2</u>	
		<u>1.4</u>	<u>1.4</u>	<u>1.5</u>	<u>1.4</u>					
		<u>0.7</u>	<u>0.7</u>	<u>0.7</u>	<u>0.7</u>					
		<u>0.6</u>	<u>0.6</u>	<u>0.6</u>	<u>0.6</u>					
No.46	<i>HindIII</i>	-	-	<u>2.5</u>	<u>2.2</u>	<u>2.2</u>	-	-	-	36A
No.73	<i>HindIII</i>	-	-	<u>3.3</u>	<u>3.0</u>	<u>3.0</u>	-	-	-	36B
No.76	<i>HindIII</i>	-	-	5.5	6.5	4.5	-	-	-	36C
						3.5				
No.95	<i>HindIII</i>	-	-	5.4	5.6	5.4	-	-	-	36D
				2.5	4.3	4.6				

a: Plant material in section I of the genus have the same band pattern and plant material in section II have the same band pattern. OD has some bands common with section I, some bands common with section II and its own specific bands. Common bands are indicated by the same coloured underline.

b: see Section 3.5. for bands not revealed in Fig. 34.

CHAPTER 9. Characterising hybrids with DNA probes

1. Introduction

There is a risk of self-pollination when attempting cross-pollination between *Zantedeschia* species because complete emasculation is difficult. In *Z. aethiopica* (section I of the genus), female flowers are interspersed with staminodes in the lower part of the spadix (Letty 1973). Generally, the stigma has lost pollen receptivity when the pollen is released from the male flowers in the same spadix. Nevertheless a few stigmas may receive pollen from the same spadix (New 1964). In the new species *Z. odorata* (OD), female flowers are interspersed with staminodes (Perry 1989) and the stigma remains receptive when pollen is released from the same spadix (Chapter 7). Complete emasculation is impossible in *Z. aethiopica* or *Z. odorata*. In section II of the genus *Zantedeschia*, upper female flowers are interspersed with a few staminodes and the stigmas are receptive to pollen from the same spadix (New 1964). Complete emasculation may not always be achieved. Therefore, identification of hybrids from the progeny of interspecific pollinations is important in this genus.

Most plants rescued from the crosses between section I and section II of the genus *Zantedeschia* were albino (Chapter 6). Plants from interspecific crosses involved in OD were either virescent, chimeric or albino (Chapter 7). It was clear that there was incompatibility between plastome and genome in these interspecific hybrids. It was interesting to know what plastome was present and whether there was any plastome mutation in these hybrids.

2. Material and methods

Plants produced from interspecific hybridisation and described in Chapter 6 and Chapter 7 were used for hybrid identification and ptDNA characterisation. All these plants were generated from the embryo axis. Besides these plants, two albino hybrids (No.43 and No.47) of CHR X CHI from a previous study (Chi 1990) were used. Multiple albino shoots were derived from both the embryo axis and the haustorium of these two hybrid embryos. Shoots derived from the embryo axis and from the haustorium were separated in tissue culture and in DNA preparations. Ten intraspecific hybrids of AE

X CHI, generated from seeds of one inflorescence, were also checked with a ptDNA probe.

Zantedeschia ptDNA clones pZAC1 and pZAC2, and several kiwifruit ptDNA clones were used for ptDNA detection. These clones are also described in Chapter 8. Two nuclear DNA probes, pZAC3 and 18s rDNA of apple, were used for hybrid identification. The characteristics of these two probes are described in Chapter 8. The rDNA probe can identify a hybrid by demonstrating that it contains bands derived from both parents. The species-specific clone pZAC3 can identify a hybrid with *Z. aethiopica* as the male parent by demonstrating the presence of bands derived from the male parent.

For hybrid identification and ptDNA characterisation, total DNA was isolated from leaf tissue. Following restriction enzyme digestion and gel electrophoresis, DNA fragments were transferred to a nitrocellulose membrane (BAS85, Schleicher & Schuell). The membrane (Southern blot) was probed and reprobed with chemiluminescence-labelled nuclear DNA or ptDNA clones (using 0.3 M NaCl in the hybridisation buffer). Detailed methods of DNA isolation, restriction enzyme digestion, gel electrophoresis, and Southern hybridisation are described in Sections 2.1., 2.8.5., 2.8.6. and 2.6.3. of Chapter 8.

3. Results

3.1. Hybrid identification by nuclear DNA probes

From the progeny of crosses between *Z. aethiopica* (AE, or CHI) and EL or G (Chapter 6), 15 green plants were checked with the rDNA probe. None of them showed the rDNA bands for the male parent and there was therefore no evidence for hybridity (Table 33). Many other green progeny of crosses between *Z. aethiopica* (female parent) and plants in section II of the genus were grown during this project. All the green plants had the morphology and flower colour of *Z. aethiopica*. It was concluded that they were all the result of self-pollination.

One albino progeny of G X CHI (Chapter 6) and two albino progeny of CHR X CHI (Chi 1990) were tested with a pZAC3 probe. All of these progeny contained the DNA sequences of the male parent and their hybridity was therefore confirmed. The

hybridty of two progeny of CHR X CHI was also supported by the demonstration of biparental ptDNA inheritance (Figs. 42 and 43).

From AE X EL (Chapter 6), nine albino progeny were tested with the rDNA probe and all of them showed the 2.0 kb rDNA band common to both parents and the 4.7 kb band inherited from the maternal parent (Fig. 39). Four progeny (No.1, 2, 5, 6) showed the 5.0 kb band from the paternal parent but the 4.0 kb band of the paternal parent was not detected (Fig. 39A). Progeny No.7 showed only a very weak 4.0 kb band but not the 5.0 kb band of the male parent after a longer exposure (Fig. 39B). The remaining four progeny (No.3, 4, 8, 9) did not show any paternal band even though a band at 5.2 kb and several bands at higher molecular weight were detected in progeny 3 and 8 after the longer exposure (Fig. 39B). These higher molecular weight bands were also detected in progeny No.1, 2, 6 and 7, but not in either parent (Fig. 39B).

From CHI X EL, four albino progeny were tested and showed the 2.0 kb common band and the 3.7 and the 4.7 kb maternal bands (lane 6, 7, 8, 9 in Fig. 40A). The 3.7 kb maternal band was weaker in lane 7 than in other lanes. Two progeny (lane 7, 8) showed the 5.0 kb paternal band but not the 4.0 kb paternal band. In addition, rDNA from both parents was detected in two albino progeny of G X AE_{sp} (Table 33).

From *Z. aethiopica* (AE, AE_{sp}, CHI) X OD, 30 progeny (Table 33) were tested with the rDNA probe and all of them were found to contain rDNA from both parents. Five progeny of AE_{sp} X OD had the 2.0 kb common band and the 4.7 kb band from the maternal parent AE_{sp} and the 6.0 kb band from the paternal parent OD (lane 1-5, Fig. 40A). The 3.7 kb band which was common to the two parents was not detected but a new band at approximately 9 kb was detected in these five hybrids. Eighteen progeny of AE X OD showed rDNA bands from both parents (Fig. 41). A variation in band intensity was observed. Lanes 1 and 7 had a weaker 2.0 kb band while lanes 2, 3, 4, 8, 9, and 13 had a weaker 6.0 kb band and a weaker 4.7 kb band. Seven virescent progeny of the cross CHI X OD were also shown to be hybrids using the rDNA probe (Table 33).

Seven progeny of OD X CHI were tested with the rDNA probe. All of them demonstrated rDNA bands from maternal and paternal parents. Moreover, 2 plants of the cross OD X AE showed biparental rDNA. These plants were therefore hybrids (Table 33).

Twenty-six green progeny of OD X CHR did not show any paternal rDNA bands. These plants are not considered to be hybrids (Table 33). A small number of albino shoots of OD X CHR grew in culture but there was insufficient material for DNA isolation.

Table 33. A summary of the results of hybrid identification

cross	No. of plants	leaf colour	hybridity		Fig.
			yes	no	
AE X EL	3	green	0	3	-
AE X G	6	green	0	6	-
CHI _{4x} X EL	5	green	0	5	-
CHI X EL	1	green	0	1	-
AE X EL	9	albino	5	4	39
CHI X EL	4	albino	2	2	40A
G X CHI*	1	albino	1	0	-
CHR X CHI*	2	albino	2	0	-
G X AE _{sp}	2	albino	2	0	-
AE X OD	18	virescent	18	0	41
CHI X OD	7	virescent	7	0	-
AE _{sp} X OD	5	virescent	5	0	40A
OD X CHI	3	virescent	3	0	-
OD X CHI	2	albino	2	0	-
OD X CHI	2	chimera	2	0	-
OD X AE	2	virescent	2	0	-
OD X CHR	26	green	0	26	-

*: For these two crosses, Southern blots of *Hind*III-digested total DNA were probed with labelled pZAC3. For the remaining crosses, Southern blots of *Eco*RI-digested total DNA were probed with labelled rDNA.

Fig. 39. Identification of *Z. aethiopica* (AE) X *Z. elliotiana* (EL) hybrids with an rDNA probe on a Southern blot

Total DNA of nine albino progeny (lane 1 to lane 9) of AE X EL, as well as AE and EL was digested with *EcoRI*. The resulting DNA fragments were separated on a 0.8% agarose gel and transferred to a nitrocellulose membrane. The membrane was probed with apple 18s rDNA. M represents the 1 kb DNA markers (BRL). The film was exposed for two minutes in (A) and one hour in (B). The triangle indicates a weak band which corresponds to the 4.0 kb band of the paternal parent (EL).

kb

M 1 2 3 4 5 6 7 8 9 AE EL

M 1 2 3 4 5 6 7 8 9 AE EL

12.2

6.1

5.1

4.1

3.0

2.0

A

B



Fig. 40. Identification of hybrids and detection of ptDNA inheritance in *Z. aethiopica* (AE_{sp}) X *Z. odorata* (OD) and *Z. aethiopica* 'Childsiana' (CHI) X *Z. elliottiana* (EL)

Total DNA of *Zantedeschia* plants was digested with *Eco*RI. The resulting DNA fragments were separated on a 0.8% agarose gel and transferred to a nitrocellulose membrane. The membrane was probed with apple 18s rDNA (A) and reprobbed with pZAC1 (B).

(1) to (5): five virescent plants of AE_{sp} X OD

(6) to (9): four albino plants of CHI X EL

(M): 1 kb DNA markers.

AE_{sp} and CHI showed the same bands of AE, which is not shown. A weak 3.7 kb band in lane 7 (A) was seen on the film but does not show up in this photograph.

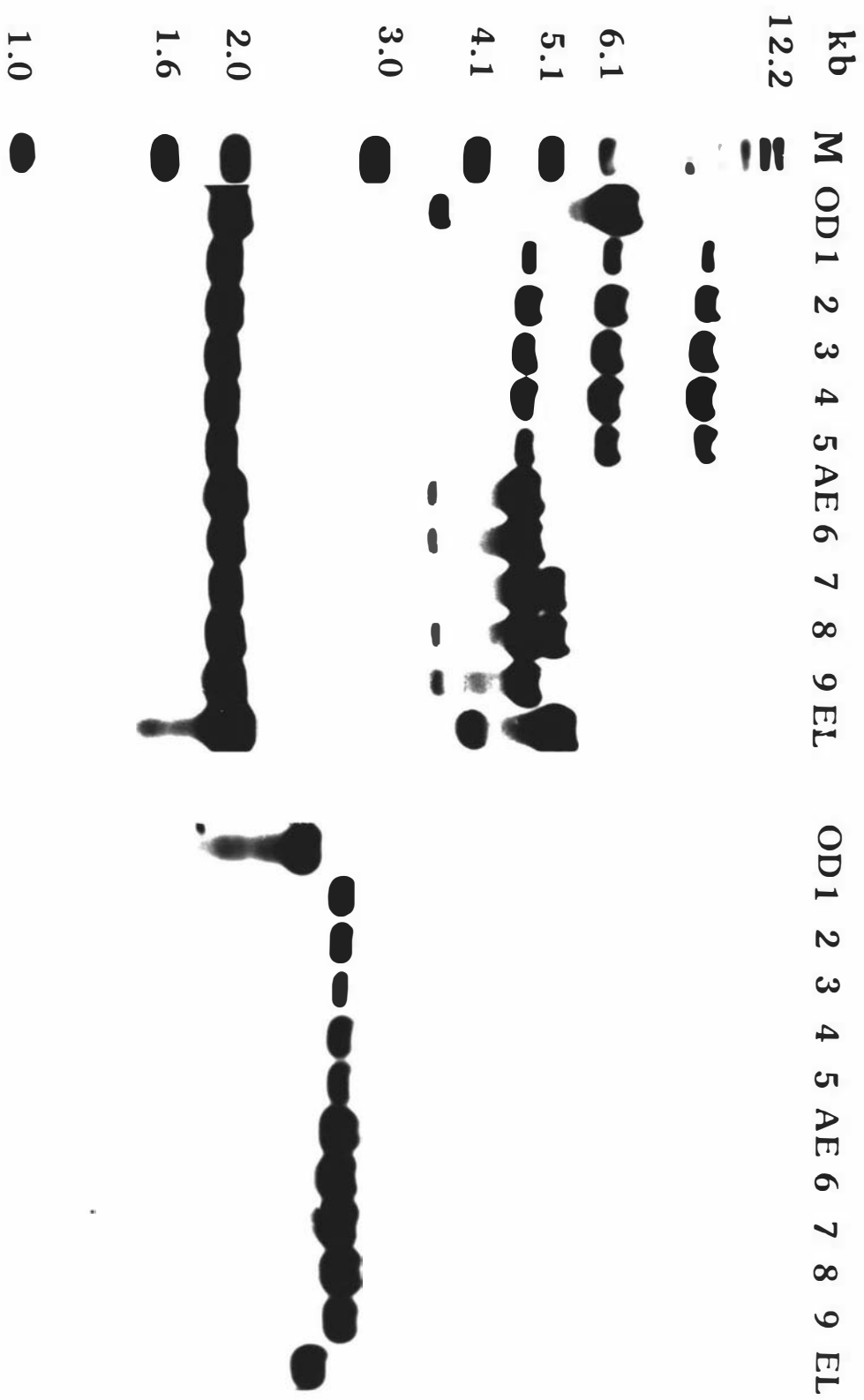


Fig. 41. Identification of *Z. aethiopica* (AE) X *Z. odorata* (OD) hybrids with an rDNA probe on a Southern blot

Total DNA of 18 virescent plants of AE X OD, (1) to (18), as well as AE and OD was digested with *EcoRI*. The resulting DNA fragments were separated on a 0.8% agarose gel and transferred to a nitrocellulose membrane. The membrane was probed with apple 18s rDNA.

Weak bands at 4.7 kb in lanes 2, 3, 4, 9 and 13, and at 6.0 kb in lane 3 were seen on the film but do not show up in this photograph.

MAE1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 ODM kb



3.2. Plastid DNA inheritance and ptDNA deletion in hybrids

A Southern blot of *Hind*III-digested total DNA of CHR, CHI, and CHR X CHI hybrids No.43 and No.47 were probed and reprobed with pZAC2 and kiwifruit ptDNA probes 1, 2 and 3. A deletion of ptDNA corresponding to kiwifruit probe 1 and possibly a part of pZAC2 was detected in No.43 (Fig. 42). Biparental ptDNA in No.47 were demonstrated. The band pattern of kiwifruit probe 2 and probe 3 in hybrid No.43 did not match well with the band pattern in the maternal parent CHR (Fig. 42). It seemed that some structural rearrangements in this area occurred. Kiwifruit probe 1 and pZAC2 were in the inverted repeat region and a part of the small single copy region of the plastome (Fig. 38). Deletion of DNA corresponding to these two probes indicated that the small single copy region and the two inverted repeats were deleted in the plastome of hybrid No.43.

It was known that DNA of No.43 was extracted from shoots with haustorial origin and DNA of No.74 was extracted from shoots with embryo-axis origin in the above experiment. It was considered that difference of ptDNA might be present between haustorial shoots and embryo-axis shoots from the same embryo. Therefore, a Southern blot was prepared with *Hind*III-digested four DNA samples of haustorial shoots and embryo-axis shoots of No.43 and No.47. When the Southern blot was probed with pZAC1, the embryo-axis shoots of both No.43 and No.47 showed biparental ptDNA. However, haustorial shoots of No.47 only demonstrated maternal ptDNA and haustorial shoots of No.43 showed a deletion (Fig. 43). It was known that pZAC1 is located within kiwifruit ptDNA probe 1 in the plastome (Fig. 38). Therefore this result confirmed the result in the above experiment and further demonstrated the difference of ptDNA between different types of shoots from the same embryo.

Nine albino progeny of AE X EL were tested with pZAC1, pZAC2, kiwifruit ptDNA probes 2 and 3 in Southern hybridisations. These four probes gave the same results on ptDNA inheritance of the nine albino plants. The hybridisation result with pZAC1 is presented in Fig. 44. Four plants (No.4, 5, 7, 9) show biparental ptDNA bands but the remaining five plants show only the maternal bands. In plant No.7 the paternal bands are stronger than the maternal bands whereas in plants No.4, 5 and 9, the maternal bands are stronger than the paternal bands (Fig. 44). These four probes did not detect any deletion in the nine albino progeny. These plants were all derived from embryo axis. Four albino progeny of CHI X EL were checked by probe of pZAC1 and

all demonstrated maternal ptDNA only (Fig. 40B). One of two albino hybrids of the cross G X AE_{sp} had biparental ptDNA (Table 34).

In contrast to the albino hybrids, only maternal ptDNA was detected in green and virescent hybrids (Table 34). With pZAC1, five virescent hybrids of AE_{sp} X OD showed only the maternal ptDNA band (Fig. 40B). In further work, eighteen virescent hybrids of AE X OD and seven virescent hybrids of CHI X OD also showed only the maternal ptDNA band with pZAC1 (Table 34). On a Southern blot, pZAC2 can distinguish two genotypes (AE and CHI) of *Z. aethiopica* by detecting a 1.4 kb *EcoRI* band of AE and a 1.5 kb *EcoRI* band of CHI (Table 32). On a Southern blot with *EcoRI*-digested total DNA, ten green intraspecific hybrids of AE X CHI were tested with pZAC2. All ten hybrids had the same ptDNA bands as those of the maternal parent AE. The 1.5 kb fragment, characteristic of CHI was not detected in any hybrid (Table 34).

Table 34. A summary of the results of plastid DNA inheritance

cross	No. of plants	leaf colour	probe	enzyme	ptDNA inheritance*			Fig.
					M	M/p	P/m	
CHR X CHI	2	albino	pZAC1	<i>HindIII</i>	0	2	0	43
G X AE _{sp}	2	albino	pZAC1	<i>EcoRI</i>	1	1	0	-
AE X EL	9	albino	pZAC1	<i>HindIII</i>	5	3	1	44
CHI X EL	4	albino	pZAC1	<i>EcoRI</i>	4	0	0	40B
AE _{sp} X OD	5	virescent	pZAC1	<i>EcoRI</i>	5	0	0	40B
AE X OD	18	virescent	pZAC1	<i>EcoRI</i>	18	0	0	-
CHI X OD	7	virescent	pZAC1	<i>EcoRI</i>	7	0	0	-
AE X CHI	10	green	pZAC2	<i>EcoRI</i>	10	0	0	-

*: M - Maternal ptDNA only. M/p - Predominantly maternal ptDNA with a lower amount of paternal ptDNA. P/m - Predominantly paternal ptDNA with a lower amount of maternal ptDNA.

Fig. 42. Biparental ptDNA inheritance and ptDNA deletion in albino hybrids of 'Chromatella' (CHR) x *Z. aethiopica* 'Childsiana' (CHI)

Total DNA of *Zantedeschia* plants was digested with *Hind*III. The resulting DNA fragments were separated on a 0.8% agarose gel and transferred to a nitrocellulose membrane. The membrane was probed with:

- (A): pZAC2
- (B): kiwifruit ptDNA probe 1
- (C): kiwifruit ptDNA probe 2
- (D): kiwifruit ptDNA probe 3
- (1): CHI
- (2): hybrid No.43 (haustorial origin)
- (3): hybrid No.47 (embryo axis origin)
- (4): CHR

Hybrid No.43 showed a deletion with kiwifruit ptDNA probe 1. Hybrid No.47 showed strong maternal ptDNA bands and weak paternal ptDNA bands.

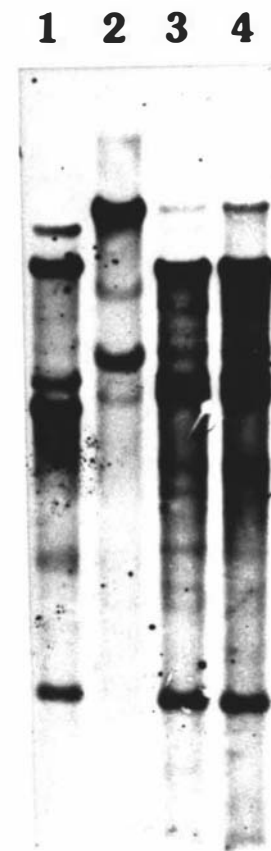
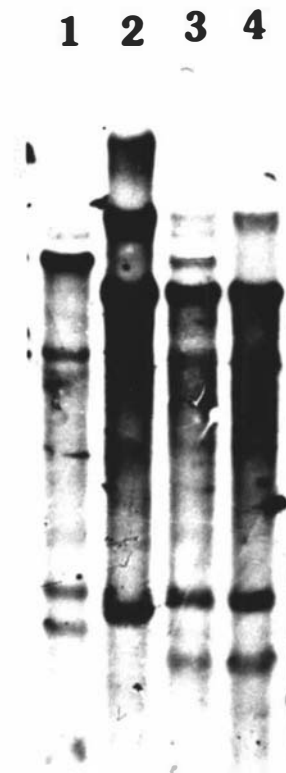
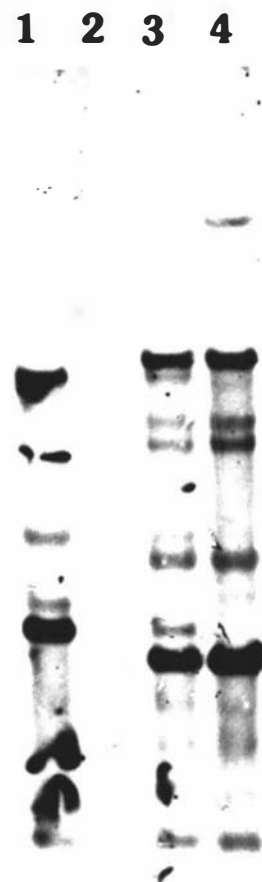
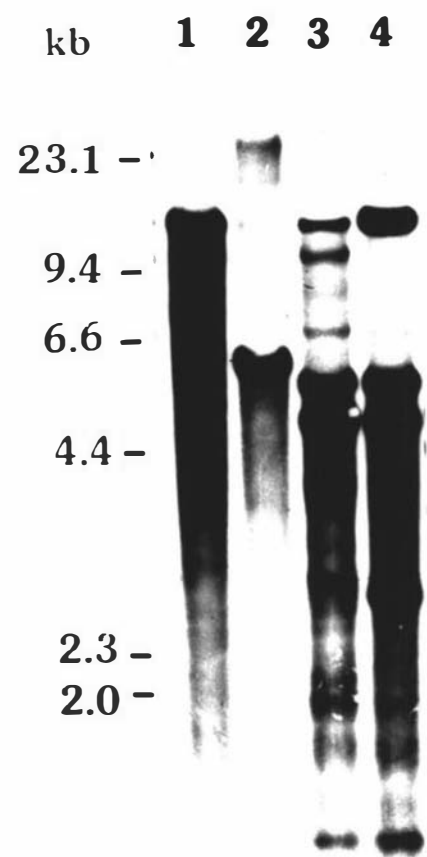


Fig. 43. Differences in ptDNA between embryo-axis shoots and haustorial shoots of hybrids No.43 and No.47 of 'Chromatella' (CHR) X *Z. aethiopica* 'Childsiana' (CHI)

Total DNA of *Zantedeschia* plants was digested with *Hind*III. The resulting DNA fragments were separated on a 0.8% agarose gel and transferred to a nitrocellulose membrane. The membrane was probed with pZAC1.

- (1): CHI
- (2): haustorial shoots of hybrid No.43
- (3): embryo-axis shoots of hybrid No.43
- (4): haustorial shoots of hybrid No.47
- (5): embryo-axis shoots of hybrid No.47
- (6): CHR

The embryo-axis shoots of both hybrids showed a biparental ptDNA inheritance. The haustorial shoots of hybrid No.43 showed a deletion. The haustorial shoots of hybrid No.47 showed maternal ptDNA only.

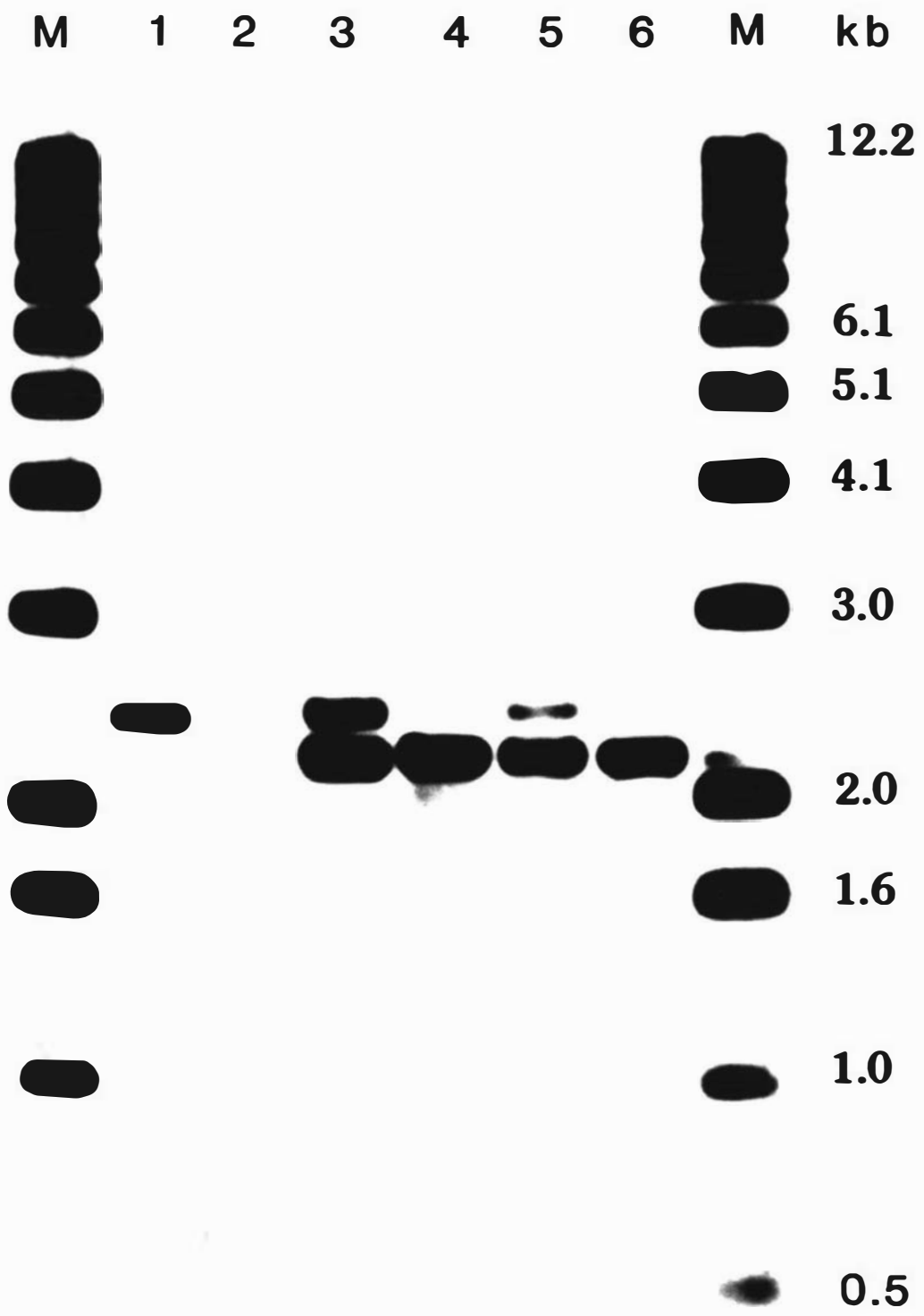
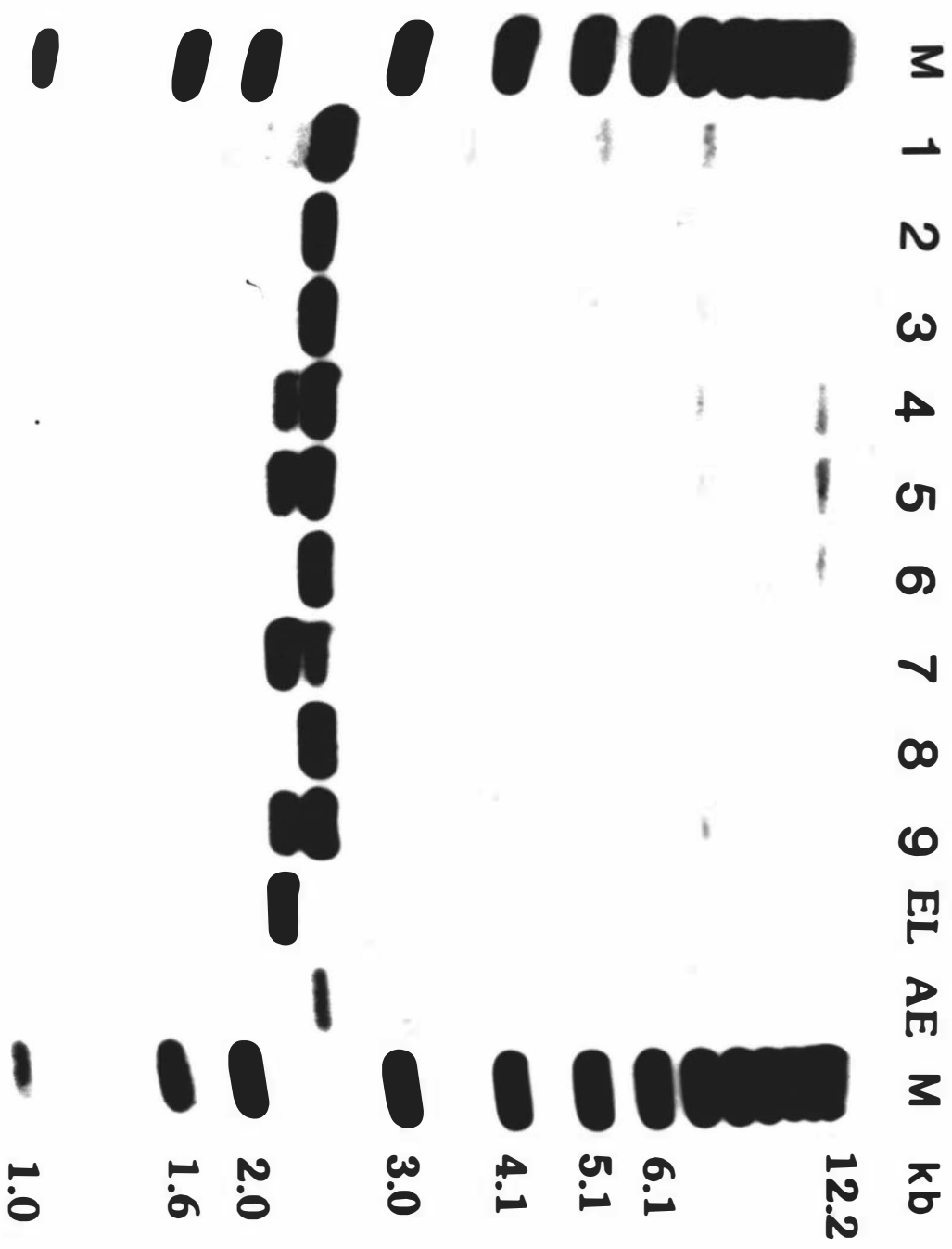


Fig. 44. Biparental ptDNA inheritance in albino hybrids of *Z. aethiopica* (AE) X *Z. elliottiana* (EL)

Total DNA of nine albino hybrids of AE X EL (lane 1-9), as well as AE and EL were digested with *Hind*III. The resulting DNA fragments were separated on a 0.8% agarose gel and transferred to a nitrocellulose membrane. The membrane was probed with pZAC1.

Hybrids No.4, 5, 7, and 9 showed biparental ptDNA inheritance but the rest of the hybrids showed only maternal ptDNA band. In hybrid No.7, the paternal band was stronger than the maternal band whereas the paternal band was weaker than the maternal band in hybrids No.3, 4 and 9.



4. Discussion

When a Southern blot was probed with rDNA, variation was found in the relative density of bands within lanes (see Fig. 41 for example). This may be caused by a variation in copy number for some of the rDNA repeat units. Rogers & Bendich (1987) reported a ten-fold variation in rDNA copy number among individuals of one species or among tissues of the same individual. Several rDNA repeat units are normally present in a plant with a variation in the intergenic spacer length. Copy number variation of some repeat units may account for the variation in bands density found in this work.

Some albino hybrids showed one paternal rDNA band but not the other paternal band (see lanes 7 and 8 of Fig. 40A for example). This may account for copy number variation (reduction) of one rDNA repeat unit as described in the above paragraph. The Southern hybridisation condition used in the present study may not detect DNA fragments with a very low copy number. Other factors may also cause the absence of one paternal rDNA bands in these albino hybrids. Reduction in the rDNA units was observed in callus-derived wheat plants (Breiman *et al.* 1987), protoplast-derived potato plants (Landsmann & Uhrig 1985) and regenerated triticales plants (Brettell *et al.* 1986). This reduction was believed to be caused by somaclonal variations.

In Fig. 40A, the 3.7 kb band normally detected in both parents, OD and AE, was not detected in their five hybrids (lane 1, 2, 3, 4, 5). In these lanes, an additional 9 kb band was detected. A possible reason for the disappearance of a smaller-sized band and the addition of a larger-sized band could be incomplete digestion caused by DNA methylation. During the course of tissue culture, a specific *EcoRI* site might become methylated. Changes in digestion patterns caused by methylation has been observed in other tissue culture studies (Phillips *et al.* 1990). In Fig. 39, larger-sized bands were detected in the albino plants even though these bands were not detected in the green parents. These bands could also be the result of incomplete digestion.

Five of nine albino plants in Fig. 39 and two of four albino plants in Fig. 40 were confirmed as hybrids by rDNA analyses. It was assumed that at least some, if not all, of the remaining six plants were hybrids because albinos were never found in the progenies of self-pollinations in *Zantedeschia* species. The nine plants in Fig. 39 were also tested with ptDNA probes (Fig. 44). Plants No.4 and No.9 (in lanes 4 and 9 of Fig. 44) demonstrated biparental ptDNA bands although they did not show paternal rDNA bands. Biparental ptDNA bands supported the hybrid status of these two plants and

implied paternal rDNA elimination or mutation. Biparental ptDNA inheritance may not be the case in all hybrids. Thus other albino plants may still be hybrids even though they do not show the rDNA or ptDNA bands of the paternal parent. Other DNA markers or isozyme markers are needed to verify this point. This feature of the results with the rDNA probe indicates that the detection of species-specific bands is a good evidence of hybridity but the absence of a band can not be used as the sole evidence to exclude hybridity.

Plastid DNA inheritance in *Zantedeschia* was investigated for the first time in this study. Of 17 albino plants from crosses CHR X CHI, G X AE_{sp} and AE X EL, seven plants showed biparental ptDNA bands and ten plants showed only maternal ptDNA bands by RFLP analysis (Table 34). In angiosperm plants, maternal ptDNA inheritance has been most commonly observed. Biparental ptDNA inheritance, however, has been found by RFLP analysis in *Pelargonium* (Metzlaff *et al.* 1981) and *Medicago* (Lee *et al.* 1988). Biparental or potential biparental inheritance of ptDNA was detected in several other genera by genetic or cytological analyses (Corriveau & Coleman 1988, Smith 1989a, Harris & Ingram 1991). The percentage of progeny containing biparental ptDNA varied from species to species. If more than 5% hybrids contain biparental ptDNA, this is defined as regular biparental ptDNA inheritance whereas if less than 5% hybrids contain biparental ptDNA this is defined as occasional biparental ptDNA inheritance (Smith 1989a). In these *Zantedeschia* crosses, more than 5% hybrids contained biparental ptDNA. Therefore regular biparental ptDNA inheritance was detected in CHR X CHI, G X AE_{sp} and AE X EL.

Biparental ptDNA inheritance in these crosses indicated that CHI, AE_{sp} and EL were capable of maintaining ptDNA in generative cells and transferring it to eggs in the course of fertilisation. However, no paternal ptDNA was detected in the ten hybrids of AE X CHI or four hybrids of CHI X EL (Table 34). It has been noted that plastids of one parent may not be maintained in a seedling or a part of a seedling even though plastids of both parents were initially present in the zygote. Eliminating plastids of one parent is known as plastid "sorting out" (Kirk & Tilney-Bassett 1978). Plastid sorting out possibly occurred in these hybrids. Different maternal parents might have different effects on the inheritance of paternal ptDNA. This phenomenon has been observed in *Medicago sativa* (Masoud *et al.* 1990). The number of plants of the crosses AE X CHI and CHI X EL examined for ptDNA inheritance were small. If more hybrids of these two crosses were examined, it might have been possible to detect biparental ptDNA inheritance. In crosses involving OD as the paternal parent, no

evidence of paternal ptDNA inheritance^{was seen} (Table 34). This could be the result of ptDNA sorting out in the seedlings but ptDNA elimination in the generative cells of OD can not be ruled out.

The control of biparental ptDNA inheritance is not clear in angiosperms but several factors are likely to be significant. The relative importance of these factors varies from one species to another. In *Petunia hybrida*, the paternal genome plays a major role on the control of biparental ptDNA inheritance (Cornu & Dulieu 1988). In *Medicago sativa*, maternal nuclear genomic influence on biparental ptDNA inheritance was observed in one study (Masoud *et al.* 1990) but the influence of both maternal and paternal genomes was observed in another study (Smith 1989b). In *Pelargonium*, ptDNA inheritance was found to be controlled predominantly by the maternal genome but the paternal genome had some effects on the ptDNA inheritance (Tilney-Bassett & Almouslem 1989). It was demonstrated that, in *Oenothera*, plastid inheritance depended on the types of plastome and the interactions between the two types of plastome contributed by both parents (Chiu *et al.* 1988).

Hybrid albinism has been shown to be the principle restriction barrier to the hybridisation between the two sections of *Zantedeschia* (Chapter 6). Biparental ptDNA inheritance in the albino hybrids indicates that^{the} plastome of each parent is incompatible with the genome of the other parent, that is, plastome/genome incompatibility is bidirectional. If only one parental plastome had been incompatible with the genome of the other parent, hybrids containing plastomes of both parents would have been at least pale green or chimeric (green/white sectors) since plastids of the second parent would have developed into chloroplasts. Change of cross direction can not overcome hybrid albinism when plastome/genome incompatibility is bidirectional. Albino hybrids have been produced from both cross directions between *Trifolium repens* and *T. hybridum* (Przywara *et al.* 1989) because of bidirectional plastome/genome incompatibility. From crosses between *T. repens* and *T. nigrescens* or *T. uniflorum* (Hovin 1962a, Pandey *et al.* 1987) albino hybrids were only produced using *T. repens* as the male parent, whereas green hybrids were produced in the reciprocal crosses. This is known as unidirectional plastome/genome incompatibility. Based on maternal ptDNA inheritance, the hybrid plastome is changed with a change in the direction of a cross. If only one parental plastome is incompatible with the genome of the other parent, albino hybrids are produced from only one cross direction.

Plastid "sorting out" can cause different parts of the same plant to have different types of plastid. This is usually evident from the plant having green and white sectors in leaves (Kirk & Tilney-Bassett 1978). Further evidence for this came from RFLP analysis (Schumann & Hancock 1989). It has been assumed that plastid sorting out takes place during early embryo development but there is no direct evidence for this. In the present work, paternal ptDNA was detected in shoots derived from embryonic axis but not in shoots derived from haustorium of hybrid embryos No.43 and No.47. of CHR X CHI (Figs. 42 and 43). As DNA samples were prepared from leaf tissue pooled from a number of shoots with the same origin, the elimination of paternal ptDNA probably occurred in the haustorium before the formation of adventitious shoots. It is possible that plastid sorting out took place during embryo development stages and resulted in the difference in plastid composition between haustorium and embryo axis of the same embryo following interspecific *Zantedeschia* hybridisation. This result may provide the first evidence for plastid sorting out within an embryo.

A deletion of ptDNA was detected in albino shoots derived from the haustorium of hybrid embryo No.43. The mechanism of this deletion is not clear. Large scale ptDNA deletions have been observed in albino plants regenerated from anther cultures of wheat and barley (Day & Ellis 1984, 1985), rice (Harada *et al.* 1991) and in albino plants derived from scutellar calli of *Hordeum marinum* (Shimron-Abarbanell & Breiman 1991). In a number of studies, ptDNA in green plants has been found to be highly conserved in the course of tissue culture (Harada *et al.* 1991, Kelmbel *et al.* 1988, Rode *et al.* 1985). The ptDNA deletion of these plants may relate to the albinism in some way. A nuclear DNA sequence has been found to be present in the embryo but absent from the endosperm of wheat grains (Tomaszewski *et al.* 1991). This deletion in the endosperm was assumed to accompany cell differentiation in developing wheat grains.

CHAPTER 10. General discussion

Most *Zantedeschia* cultivars have been bred following interspecific hybridisation within section II of the genus. *Z. aethiopica* possesses several desirable characters lacking in section II and vice versa. Intersectional hybridisation is attractive to plant breeders because there is a great potential to create improved cultivars through combinations of complementary characters from both sections. However, early studies did not achieve any hybrids between the two sections (Traub 1949) and no successful intersectional crosses have been reported since then. In a recent report (Chi 1990), the main barrier to the intersectional crosses appears to arise from postfertilisation incompatibility.

Initial plans in the present study were to test the effects of different species and ploidy levels on the crossability between the two sections and to develop a method for hybrid identification. Embryo culture was planned to rescue hybrid embryos. When all rescued hybrid embryos were found to be albinos, one of the research interests focused on characterising the plastomes of the hybrids. In the middle of this study, an intermediary species, *Z. odorata*, became available and 'bridge' hybridisation was attempted.

Species relationships sometimes offer clues for parent selection in interspecific hybridisation. Chromosome analysis has been widely applied in species-relationship studies (Hsiao *et al.* 1986), but not previously exploited in the genus *Zantedeschia*. The chromosome karyotype analysis in Chapter 4 demonstrated two distinct karyotypes corresponding with the two sections of the genus in Letty's classification (1973) based on morphological characters. The new species, *Z. odorata*, possesses a karyotype distinguishable from both sections. According to karyotype asymmetry, *Z. odorata* was placed between the two sections but more closely related to *Z. aethiopica*. Describing morphological characters, Perry (1989) suggested that *Z. odorata* may be an intermediate between the two sections. These results encouraged us to use *Z. odorata* as a bridge species for the gene transfer between section I and section II. As karyotypes in section II were not distinct between species, an attempt to select a species more closely related to section I for hybridisation was not possible. The species relationships determined by karyotype analysis are confirmed by the interspecific hybridisation results in Chapter 6 and Chapter 7.

Tetraploid plants have been used to overcome interspecific incompatibility in some genera. Only diploid plants were used in interspecific hybridizations in *Zantedeschia*. To test the effects of ploidy level on intersectional crossability in *Zantedeschia*, tetraploid plants have been produced from both sections. Buds of plants in section II can be readily multiplied *in vitro* (Cohen 1981). By incorporating colchicine treatment into a shoot multiplication system, tetraploids have been produced from eight cultivars and two species. Buds of *Z. aethiopica* did not multiply readily *in vitro*. For this species, colchicine treatment of germinating seeds was used for tetraploid production. Plants generated after colchicine treatment were screened as putative tetraploids using stomatal measurements. Subsequently, tetraploids have been confirmed by chromosome counting. Most of ^{the} tetraploid plants produced *in vitro* were pure tetraploids but most of ^{the} tetraploid plants produced from germinating seeds were chimeras. Tetraploid production *in vitro* showed not only an advantage in reducing the frequency of chimeras but also in increasing the efficiency of tetraploid production compared to colchicine treatment of seeds. The *in vitro* method may be used to double the chromosome number of hybrids for improving fertility and adapted to other plant species for which a micropropagation method is available.

In the intersectional hybridisations, postfertilisation incompatibility caused endosperm degeneration and inhibited embryo development. The hybrid endosperms were watery and transparent after six weeks from pollination because of cellular degeneration. Most hybrid embryos did not develop beyond the globular stage. Using different genotypes from the two sections did not alter the development of the endosperms and embryos *in vivo*. The use of tetraploid plants as one parent did not improve the development of the endosperms and embryos. When embryos were too small to be separated from the endosperm, an alternative embryo culture technique was used for hybrid embryo rescue. The embryos imbedded within the endosperm were initially cultured. From 2500 initially cultured embryos, approximately 200 were rescued and formed hybrid shoots, and in some case formed roots. Unfortunately, all hybrids were albino and can survive only on artificial medium. Cross direction and different genotypes showed differences in the recovery of albino hybrids but did not show any difference in the hybrid albinism. Although the tetraploids of 'Childsiana' were later shown to be chimeric, it can be concluded that tetraploid plants from section II and possibly section I used as one parent do not improve the recovery of intersectional hybrids.

To exploit an alternative way for gene recombination between section I and section II, an intermediary species, *Z. odorata*, was attempted as a 'bridge' for gene flow between the sections. Hybridisation between *Z. odorata* and *Z. aethiopica* produced a number of virescent, albino and chimeric (green/albino) hybrids. Although embryo rescue was used early in this study, seeds from these crosses also germinated. Hybrid production was much easier when *Z. aethiopica* was used as the maternal parent. Crosses between *Z. odorata* and 'Chromatella' or *Z. rehmannii* produced a number of elongated embryos, i.e., embryos larger than 1 mm, but all resulting hybrids were albino. The barrier of poor-embryo development appears to be at least partly overcome. However, the barrier of plastome/genome incompatibility remains. The role of *Z. odorata* as a bridge for gene flow will only be achieved after viable hybrids are produced between *Z. odorata* and section II. Perhaps a further study may produce green or partially green hybrids between *Z. odorata* and other genotypes from section II.

Although the embryo development was poor *in vivo* following cross-pollination between section I and section II, a number of hybrid embryos grew and produced shoots *in vitro*. This indicates the postfertilisation incompatibility which affects embryo development *in vivo* can be overcome by the embryo culture technique used. However, hybrid albinism caused by plastome/genome incompatibility was not overcome by using a range of genotypes, changing the ploidy level of one parent or via embryo culture. It is, therefore, concluded that hybrid albinism is the most significant restriction to the hybridisations between section I and section II, and between *Z. odorata* and section II.

Studies on plastid ultra-structure and ptDNA were undertaken in an attempt to understand hybrid albinism. Plastids of albino hybrids had no prolamellar bodies when grown in the dark nor grana when grown in the light. The prolamellar body is a structure typical of normal etioplasts and grana are structures typical of chloroplasts. The absence of these structures indicates that plastid development in the albino hybrids is not only blocked in the late stages corresponding to chloroplast formation but also in the early stages corresponding to the etioplast formation. Large starch grains were found in the plastids of albino hybrids. This demonstrates that plastids of albino hybrids are functional for starch synthesis even though they are not functional for photosynthesis.

Two ptDNA clones of *Z. aethiopica* 'Childsiana', as well as eight kiwifruit ptDNA clones, were used to characterise the ptDNA in the parents and hybrids. Biparental ptDNA

were detected in four out of nine albino hybrids of *Z. aethiopica* X *Z. elliotiana*. This result indicated that the plastome of each parent was incompatible with the genome of the other parent. In other words, the plastome/genome incompatibility between *Z. aethiopica* and *Z. elliotiana* was bidirectional. Thus even if hybrids had been rescued from the reciprocal cross, *Z. elliotiana* X *Z. aethiopica*, they might have been albino. Differences in ptDNA were detected between albino shoots derived from the embryo axis and those from the haustorium of the same hybrid embryo. The paternal ptDNA elimination in the haustorium but not in the embryo axis of the same embryo may be caused by ptDNA sorting out. No paternal ptDNA was detected in green intraspecific hybrids of *Z. aethiopica* or virescent interspecific hybrids of section I X *Z. odorata*. From crosses *Z. odorata* X *Z. aethiopica* 'Childsiana', virescent, chimeric and albino hybrids were produced. The ptDNA in these hybrids has not yet been studied. It would be interesting to determine whether virescent leaves contain different ptDNA from albino leaves.

Although reciprocal hybrids between two genotypes (CHI and AE) of *Z. aethiopica* are always green, the plastome/genome interactions between *Z. odorata* and these two genotypes are different (Chapter 7). There is at least one difference between the plastomes of CHI and AE as revealed by ptDNA probe pZAC2 (Table 32). This difference(s) may contribute to the different plastome/genome interactions observed. Plastome variations may be present among species or genotypes of section II even though these were not detected by the restriction enzymes and probes used in this study. Using other species and genotypes from section II may produce green or virescent hybrids with *Z. odorata*. A further study may detect the differences among the plastomes from section II by using more combinations of restriction enzymes and ptDNA probes in RFLP analysis. This RFLP data may indicate plastome relationships in the genus and further reveal a plastome from section II closely related to plastome of *Z. odorata*. Using this type of plastome should help overcome hybrid albinism.

Albino hybrids have been produced from interspecific hybridisations in many other genera, such as *Trifolium* (Hovin 1962a, Pandey *et al.* 1987, Przywara *et al.* 1989), *Impatiens* (Arisumi 1985) and *Oenothera* (Stubbe 1964, Kirk & Tilney-Bassett 1978). Researchers believe that plastome/genome incompatibility is the cause of hybrid albinism. Five types of plastome and six types of genome were identified in the subgenus *Euoenothera* of *Oenothera* (Stubbe 1964, Kirk & Tilney-Bassett 1978). Some plastomes were compatible with several genomes whereas in others they were compatible with only one or two genomes. In some interspecific *Trifolium* crosses,

albino hybrids were produced from only one crossing direction (Hovin 1962a, Pandey *et al.* 1987). This indicated, based on maternal plastid inheritance, that the plastome of one parent was compatible with the genome of both parents but the plastome of the other parent was compatible only with its own genome. The genetic basis for hybrid albinism is not fully understood in any genus.

There are further examples of albino plant production from pollen derived plants. Albino plants have been produced from anther cultures of rice (Harada *et al.* 1991), wheat (Zhou *et al.* 1991) and barley (Larsen *et al.* 1991). The albinism in anther cultures of barley and wheat are mainly controlled by a few nuclear genes (Larsen *et al.* 1991, Tuveesson *et al.* 1989, Agache *et al.* 1989). Genetic control for albinism in pollen derived plants may be different from that for albinism in interspecific hybrids.

Hybrid identification is necessary in wide hybridisation and identifying hybrids at an early developmental stage is desirable. There are few clear morphological differences between *Zantedeschia* species at the young seedling stage. Chromosomes were found to be unsatisfactory in hybrid identification because all species have the same chromosome number and chromosome sizes are small. Molecular biological methods have been applied for hybrid identification in many studies (see Chapter 3 for references). These methods can provide unequivocal data on hybrid status and can identify hybrids at a very early stage. By these methods, it is also possible to determine the hybridity of callus and albino shoots. In the present study, a partial library was constructed with total leaf DNA of *Z. aethiopica* 'Childsiana'. From this library, a species-specific nuclear DNA clone, pZAC3 was isolated. This clone was used to identify hybrids where plants in section I were used as the male parents and plants in section II were used as the female parents. It was found that an apple rDNA clone could distinguish different *Zantedeschia* species by Southern hybridisation. This apple rDNA clone was successfully used to identify hybrids from all crosses with *Z. aethiopica* as the male or female parent. These DNA clones could be used for hybrid identification and hybrid genome characterisation in further *Zantedeschia* interspecific hybridisations.

The previous study (Chi 1990) pursued embryogenesis in self- and cross-pollinations and demonstrated that postfertilisation incompatibility is the main barrier for hybridisation between the two sections of *Zantedeschia*. The present study tried several ways to overcome the incompatibility and produced approximately 200 albino hybrids. Hybrid albinism is concluded to be the most significant barrier for the

intersectional hybridisation. Several other approaches have been used in other genera to overcome incompatibility, which may be used in *Zantedeschia* in further studies. For instance, it would be interesting to use pollen irradiation in an attempt for producing asymmetric green hybrids between the two sections or between *Z. odorata* and section II. Pollen irradiation technique (Shintaku *et al.* 1988) has been used to overcome sexual incompatibility between *Nicotiana repanda* and *N. tabacum* through the elimination of genes which control the incompatibility. Fusion of irradiated protoplasts with non-irradiated protoplasts has been used to overcome somatic incompatibility (Gupta *et al.* 1984, Dudits *et al.* 1987, Gleba *et al.* 1988)

In summary, this study has applied (a) cytogenetic methods to analyse species-relationships, (b) tissue culture methods to produce tetraploids and rescue hybrid embryos, (c) molecular biological methods to identify hybrids and characterise plastomes. The considerable body of information gathered here will be very useful for further studies. Although no green hybrids were obtained from the intersectional crosses due to hybrid albinism, the triploid and tetraploid plants produced, and DNA probes isolated will be very useful materials in further research.

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Appendices

Appendix 1. Key to *Zantedeschia* Species (Based on Letty 1973)

Section I:

Plants evergreen; female flowers interspersed with staminodes

1. *Z. aethiopica*

Section II:

Plants deciduous; female flowers not interspersed with staminodes:

Leaves narrowly lanceolate, cuneate at the base

2. *Z. rehmannii*

Leaves hastate to cordate at the base:

Spathes golden yellow to lemon yellow:

Leaves triangular-hastate, always maculate; spathe golden yellow

3. *Z. jucunda*

Leaves oblong-hastate to ovate-orbicular, cordate, maculate or immaculate:

Leaves ovate-orbicular; maculate; spathe without a purple blotch at base

4. *Z. elliottiana*

Leaves oblong-hastate, usually immaculate; spathe usually with a purple blotch at base

5. *Z. pentlandii*

Spathes white, cream, ivory, pale greenish-yellow or rarely coral pink:

Leaves either immaculate or conspicuously maculate, usually oblong-hastate, rarely ovate-hastate (and then white spotted), lower lobes short to triangular-spreading or strap-shaped; spathes with limb more or less spreading , usually tapering gradually to the apex

6a. *Z. albomaculata* subsp. *albomaculata*

Leaves immaculate or slightly maculate, rarely conspicuously maculate, triangular-hastate to ovate-orbicular-cordate, lower lobes bluntly triangular; spathes more or less truncate, abruptly apiculate:

Leaves usually triangular-hastate, usually slightly maculate; spathes more or less truncate; berries large (up to 2 cm diam.)

6b. *Z. albomaculata* subsp. *macrocarpa*

Leaves ovate-cordate to ovate-orbicular-cordate, immaculate; spathes large, limb somewhat spreading; berries medium-sized (up to 1 cm in diam.)

6c. *Z. albomaculata* subsp. *valida*

Appendix 2. Potting mix for growing *Zantedeschia* plants in greenhouses.

Steam sterilise 30 litres of pumice and 50 litres of peat, then add the following fertilizers:

240 grams Dolomite

40 grams Osmocote (for short term growth, approximately 4 months)

120 grams Osmocote (for long term growth, approximately nine months)

80 grams Micromax

This is the standard potting mix for growing *Zantedeschia* and *Nerine* plants in greenhouses at Horticulture and Food Research Institute of New Zealand, Palmerston North.

Appendix 3. Crosses made in three different seasons (described in Chapter 6).

1 st season		2 nd season		3 rd season	
crosses	N*	crosses	N	crosses	N
Section I X Section II					
AE X G	6	AE X EL	24	AE X G	4
AE X PPK	1	CHI X EL	78	AE X GS	2
AE X RE	1	CHI _{4x} X EL	36	CHI X G	2
AE X GS _{4x} **	4			AEsp X PPK	1
AE X BM _{4x}	1			AE X RE	2
CHI X G	2			AE X GS _{4x}	2
CHI X PP	3			AE X RE _{4x}	1
CHI X RE	3			AEsp X PPK _{4x}	1
CHI X GS	1			AE X BM _{4x}	3
CHI X BM _{4x}	4				
CHI _{4x} X BM _{4x}	1				
CHI _{4x} X PPK	2				
CHI _{4x} X G	1				
total	30		138		18
Section II X Section I					
RB X CHI	1	EL X AE	1	RQ X CHI	18
Pst X CHI	2	AE X CHI	17	RG X CHI	2
G X CHI	3	AE X CHI _{4x}	6	RE X CHI	3
GS X CHI	2			MR X CHI	1
GS X CHI _{4x}	3			RQ X AE	16
GS _{4x} X CHI	5			GS X AE	1
RQ X CHI	2			RE X AE	2
RQ _{4x} X CHI	8			RG X AE	1
RQ X CHI _{4x}	3			PPK X AEsp	1
PP X CHI	1			G X AEsp	1
PP X CHI _{4x}	1			PP X AEsp	3
BM _{4x} X CHI	1			PS X AEsp	1
PPK X CHI _{4x}	4			PST X AEsp	3
RE X CHI _{4x}	1			BG X AEsp	1
				GS _{4x} X CHI	11
				RQ _{4x} X CHI	20

--- To be continued on next page

* N: Number of inflorescences.

** Tetraploids are indicated by "4x".

continue from Appendix 3.

1 st season		2 nd season		3 rd season	
crosses	N	crosses	N	crosses	N
				RQ _{4x} X AE	7
				RE _{4x} X AE	1
				GS _{4x} X AE	1
				PPK _{4x} X AEsp	1
				BM _{4x} X AEsp	1
total	37	24			100
Section I X Section I					
CHI X CHI	4	AE X AE	1	AEsp X AE	1
AE X AE	1	CHI X CHI	3	AE X AE	3
AE X CHI	3	AE X CHI	5	AEsp X AEsp	1
CHI X CHI _{4x}	1				
AE X CHI _{4x}	1				
total	10	9			5
Section II X Section II					
GS X G	3	EL X EL	4	GS X GS	8
GS X GS	3			PPK X PPK	5
RQ X G	1			RQ X RQ	2
RQ X GS	1			RE X RE	4
PPK X PPK	2			PS X PS	2
RE X PPK	1			G X G	2
RG X PPK	1			PST X PST	1
RE X RE	2			RQ X RE	4
BM X BM	2			GS X GS _{4x}	3
GS _{4x} X GS	1			RQ X GS _{4x}	3
RQ X GS _{4x}	2			PPK X RE _{4x}	1
BM X BM _{4x}	2			GS _{4x} X GS _{4x}	3
BM _{4x} X BM _{4x}	1			RQ _{4x} X GS _{4x}	1
RQ _{4x} X CHI	1			PPK _{4x} X GS _{4x}	1
				G _{4x} X G _{4x}	3
				RE _{4x} X RE _{4x}	1
				PPK _{4x} X PP _{4x}	1
total	23	4			49
sum	100	175			172

Appendix 4. Crosses involving *Z. odorata* and control pollinations (described in Chapter 7).

section I/OD	N*.	section II/OD	N.	controls	N.
AE X OD	11	RE X OD	1	CHI X CHI	1
AEsp X OD	1	CHR X OD	14	OD x OD	8
CHI X OD	13	OD X CHR	10	CHR X CHR	2
OD X AE	1			CHI X AE	3
OD X CHI	2**			CHR X RE	1

* Number of inflorescences.

** A putative tetraploid plant of CHI (CHI-T58) produced in Chapter 5 was used in the pollination of one inflorescence.